Investigation of the Time Dependent Influence of Extracellular Osmotic Stress on Protein Turnover in Skeletal Muscle Cells

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Abstract

Acute alterations in cell volume can substantively modulate subsequent metabolism of substrates. However, how such alterations in skeletal muscle modulate protein metabolism is limited. The purpose of this study was to determine the time dependent influence of extracellular osmotic stress on protein turnover in skeletal muscle cells. L6 cells were incubated in hyperosmotic (HYPER; 425.3 ± 1.8mmol/kg), hypo-osmotic (HYPO; 235.4 ± 1.0mmol/kg) or control (CON; 333.5 ± 1.4mmol/kg) media for 4, 8, 12, or 24hrs. During the final 4hrs, incorporation of L-[ring-3,5-^{3}H]-tyrosine was measured to estimate protein synthesis. Western blotting measured markers of protein synthesis and degradation. No differences were observed in any outcomes except p70S6K phosphorylation whereby HYPO was lower ($p<0.05$) than CON and HYPER; which remained similar except for a large increase at 8hrs for HYPER. These findings suggest that regardless of duration, extracellular osmotic stress does not significantly affect protein metabolism in L6 cells.

Keywords: *cell-shrinkage, cell-swelling ,osmotic stress, protein metabolism, skeletal muscle*

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List of Abbreviations

4E-BP1- Eukaryotic Translation Factor 4E Binding Protein 1

ACE- Angiotensin Converting Enzyme

ADH- Antidiuretic Hormone

AE1- Anion Exchanger 3

AFC- 7-amino-4 trifluoromethylcoumarin

AHS- Adult Horse Serum

Akt- Protein Kinase B

AMC- 7-amino-4-methylcoumarin

ANOVA- Analysis of Variance

ANP- Atrial Natriuretic Peptide

AQP- Aquaporin

ATP- Adenosine Triphosphate

BSA- Bovine Serum Albumin

Ca^{2+}- Calcium Ion

cAMP- Cyclic Adenosine Monophosphate

CFTR- Cystic Fibrosis Transmembrane Conductance Regulator

CHIP- C Terminus of HSC70 Interacting Protein

CHO- Chinese Hamster Ovary

CO_2- Carbon Dioxide

CON- Control

CPM- Counts per Minute

DCT- Distal Convoluted Tubule

DHPR- Dihydropyridine Receptor

DMEM- Dulbecco's Modified Eagle Medium

DNA- Deoxyribonucleic Acid

E1- Ubiquitin Activating Enzyme

E2- Ubiquitin Conjugating Enzyme

E3- Ubiquitin Ligase

EDL- Extensor Digitorum Longus

eEF2K- Eukaryotic Elongation Factor 2 Kinase

eIF4E- Eukaryotic Initiation Factor 4E

FBS- Fetal Bovine Serum

GLUT1- Glucose Transporter 1

H_2O_2- Hydrogen Peroxide

HICC- Hypertonicity Induced Cation Channels

HRP- Horse Radish Peroxidase

HYPER- Hyperosmotic Stress

HYPO- Hypo-osmotic Stress

ISO- Iso-osmotic

K^+- Potassium Ion

KCC- Potassium Chloride Cotransporter

MAFbx- Muscle Atrophy F-Box

MAPK- Mitogen-activated Protein Kinase

mRNA- Messenger Ribonucleic Acid

mTOR- Mammalian Target of Rapamycin

MuRF-1- Muscle Ring Finger 1

Na^+- Sodium Ion

NHE- Sodium Proton Exchanger

NKCC- Na/K/Cl cotransporter

O_2- Oxygen

p70S6K- p70 Ribosomal S6 Kinase

PBS- Phosphate Buffered Saline

pCMBS- p-chloromercuribenzene sulfonate

PDCD4- Programmed Cell Death Protein 4

PDHα- Pyruvate Dehydrogenase Alpha

PDK- Phosphoinositide Dependent Kinase

PI3K- Phosphoinositide 3 Kinase

PIP2- Phosphatidylinositol 4,5 Bisphosphate

PIP3- Phosphatidylinositol 3,4,5 Trisphosphate

PRAS40- Proline Rich Akt Substrate of 40kDa

PTEN- Phosphatase and Tensin Homolog

PVDF- Polyvinylidene Fluoride

RAS- Renin Angiotensin System

RBC- Red Blood Cell

Rh- Rhesus Blood Factor

RNA- Ribonucleic Acid

ROS- Reactive Oxygen Species

RR- Ryanodine Receptor

RTK- Receptor Tyrosine Kinase

RVD- Regulatory Volume Decrease

RVI- Regulatory Volume Increase

SDS- Sodium Dodecyl Sulfate

SEM- Standard Error of the Mean

Ser- Serine

SKAR- S6K1 aly-REF-like Target

SOL- Soleus

TBS- Tris Buffered Saline

TBST- Tris Buffered Saline and Tween 20

TCA- Trichloroacetic Acid

Thr- Threonine

TRAF6- TNF Receptor-associated Factor 6

TRIM32- Tripartite Motif-Containing Protein 32

tRNA- Transfer Ribonucleic Acid

Chapter 1.1: Water & Hydration- Physiological Importance

I. Introduction

Water is essential for human survival and as such is one of the most abundant molecules found within the human body. In an average adult male, water will make up approximately 60-65% of total body weight, however; these values can fluctuate depending on several variables (age, gender, etc.). For example, elderly individuals generally have proportionally less water in their body (50-55%) due to the accumulation of fat over time (Popkin, D'Anci et al. 2010). Water has many vital physiological roles within the human body. These roles include transport of nutrients, removal of waste, maintenance of body temperature, a medium for reactions, and as a shock absorber and lubricant. As the body relies on water for so many of its vital functions it appears that hydration and fluid content within the body and individual cells is monitored and regulated very closely by several homeostatic mechanisms.

II. Compartmentalization and Subsequent Transport of Water

IIa. Fluid Compartments

Within the human body there is on average 42L of water, which is divided between two specific compartments, the intracellular and extracellular compartments. The intracellular fluid accounts for roughly two thirds (28L) of total body water, while the other third (14 L) is found in the extracellular fluid (Friedman 2010). The intracellular fluid consists of all the fluid within the cells of the body. Its primary role is to act as a reaction medium within the cell, allowing

for many metabolic reactions to occur. The extracellular fluid is considered all of the fluid that is not found within cells and is often separated into the following categories: plasma, lymphatic, and interstitial. The plasma is the liquid portion of blood, accounting for roughly 3L of total body water. It acts as the aqueous medium in the circulatory system and is thus responsible for transporting nutrients and waste throughout the body. Plasma allows for the mobility of red blood cells (RBCs) facilitating the exchange of gases (O_2, CO_2) at tissues and at the lungs. The largest compartment of the extracellular fluid is the interstitial space that contains interstitial fluid. This fluid surrounds and bathes the cells of the body and has many diverse roles including transportation of nutrients and waste between the circulatory system and tissue, lubrication of joints and protection of vital organs (Wiig, Rubin et al. 2003, Ateshian 2009). The final and smallest portion of the extracellular fluid is found in the lymphatic system. This system, consisting of capillaries, vessels, nodes, trunks and ducts, are responsible for the collection and subsequent one way transport of fluid and proteins from the interstitial space back to the circulatory system (Swartz 2001). The lymphatic system is also an integral part of the human immune system. Under normal conditions each of these different compartments has a distinct composition, but due to perturbations (environmental, behavioural) in homeostasis these can be altered causing both ionic and fluid shifts across the different compartments.

IIb. Osmolality

Osmolarity is a measure of solute concentration commonly used in

physiology which specifically represents the number of particles dissolved in a liter of solution, often presented as osmoles per liter (Osm/L). Due to the incorporation of a solution's volume when measuring osmolarity, it is susceptible to fluctuations which are dependent on temperature and the removal or addition of solutes. In contrast, another measurement of solute concentration is osmolality, specifically the number of particles dissolved in a kilogram of solution (Osm/kg). The weight of a solution is relatively constant across varying temperatures and the addition or removal of solute so it is a more reliable measure. However, under circumstances where the solutions are very dilute and temperature is well regulated, which is the case in the human body, the variance between osmolarity and osmolality are minimal.

In general, the fluid compartments of the human body have an osmolarity of approximately 290 mOsm/L (Kraft 2000). However, the osmolarity of extracellular fluid is often altered in response to many stressors, both environmental and behavioural. Some of these stressors include exercise, heat, diabetes, diet, and hydration status (Maughan and Shirreffs 1997, Hashim and Zarina 2012). These imbalances between intracellular and extracellular osmolarities cause fluid shifts as the body tries to return to homeostasis. Cellular membranes are considered to be permeable to water but almost completely impermeable to many solutes (Fettiplace and Haydon 1980). This means that there is rapid regulation of extracellular and intracellular osmolarity and this occurs through the movement of water and alterations in cell volume. Commonly, there are three ways to classify the extracellular osmolarity in relation to

intracellular osmolarity and the subsequent changes in cell volume. These three classifications are iso-osmotic, hypo-osmotic, and hyperosmotic.

An iso-osmotic extracellular osmotic environment occurs when the extracellular osmolarity is roughly equivalent to the intracellular osmolarity (290 mOsm/L). Under these conditions, there is no net movement of water across the cell membrane from one compartment to another. That is not to say there is no movement of water, but rather the influx and efflux of water occur at the same rate. This extracellular environment is considered to be the most favourable due to minimal regulation as compared to the other extracellular osmotic states. When there is a greater concentration of solutes per litre of solution in the extracellular fluid compared to the intracellular environment, it is said to be a hyperosmotic extracellular osmotic environment (>290 mOsm/L). The cell will attempt to maintain equivalent osmolarities across the membrane, so water will move from the compartment with a high concentration of water (intracellular) to the compartment with a low concentration of water (extracellular). This will lead to a net movement of water out of the cell and a subsequent decrease in cell volume. The final classification is a hypo-osmotic extracellular osmotic environment (<290 mOsm/L). In this case there is a higher concentration of solutes per litre of solution in the intracellular compartment than the extracellular compartment. Consequently, there will be an influx of water as the cell tries to match intracellular and extracellular osmolarities ultimately leading to the accumulation of water inside the cell and increase in cell volume. These alterations in cell volume have been shown to influence a wide array of functions

within the human body: cell proliferation as well as hormone and transmitter release and metabolism (Pendergrass, Angello et al. 1991, Bruck, Haddad et al. 1992, Haussinger, Lang et al. 1994). However, these perturbations are corrected rapidly by cellular response mechanisms that restore cell volume to their initial state (Okada 2004).

IIc. Water Transport

The transport of water across the cell membrane of human cells is vital to the homeostatic maintenance and overall function of the human body. The original consensus was that water only crossed the membranes of cells by diffusion along a concentration gradient, from an area of high to low water concentration. However, this has since been disproven with the discovery and characterization of a family of protein channels – aquaporins - which act to facilitate the diffusion of water. It is now suggested that there are three primary mechanisms by which water crosses biological membranes: diffusion, aquaporins and other transporters that are not specific for water (Haines 1994).

The osmotic permeability of cell membranes to transport water by means of diffusion has been calculated to range between 2 to 50 x 10^{-4}cm/s (Haines 1994). However, measured values of water transport across a membrane exceed the values that can be obtained from diffusion alone. This led to the hypothesis that cells must be capable of transporting water by other means, such as through a water channel. Subsequently, several candidates were identified with water transport capabilities that were thought to be water channels. A proposed candidate was the band 3 anion exchanger (AE1) found within RBCs (Solomon,

Chasan et al. 1983). Evidence supporting this possibility include a large number of copies of the band (10^6 copies/RBC) and the presence of a sulfhydryl group that is covalently modified by pCMBS (an organomercurial inhibitor of water channels). This has since been disproven due to the inconsistent inhibition by stilbene analogues and also the transport of water by organomercurials (Macey and Farmer 1970). More supporting evidence against AE1 being a water channel included a lack of water and anion transport when oocytes were expressed with AE1 (Zhang, Alper et al. 1991). Other potential water channels identified were the sodium independent glucose carrier (GLUT1) and the cystic fibrosis transmembrane regulator (CFTR). GLUT1 has been shown to transport not only its substrate (glucose) but also water, leading to the postulation that it may be a water channel (Fischbarg, Kuang et al. 1989, Fischbarg, Kuang et al. 1990). However, since this postulation there has since been a consensus that it is not a water channel (Dempster, van Hoek et al. 1991, Zhang, Alper et al. 1991, Zeidel, Ambudkar et al. 1992). CFTR also showed the capacity to transport water as well as anions and small solutes when stimulated with cAMP (Hasegawa, Skach et al. 1992). Indirect measurements have suggested that the permeability of CFTR to water is quite large but direct measurements have yet to be conducted. Therefore it seems that both GLUT1 and CFTR are capable of transporting water but it is not yet known whether the amount transported is of physiological significance.

The discovery of the first water channel, known as channel-like integral protein of 28 kDa (CHIP28) (now known as aquaporin 1 (AQP1)), was first

identified by means of a coincidence. The Agre lab was conducting studies on the rhesus (Rh) blood group antigens and through the use of hydroxylapatite chromatography found a protein that was 32 kDa (Agre, Saboori et al. 1987, Saboori, Smith et al. 1988). Experiments were then conducted to detect the protein using silver staining, however another protein was found at 28 kDa originally thought to be a fragment of the initial 32 kDa protein. After further characterization, these two proteins were confirmed to be two completely different proteins as a specific antibody would only bind to the 28 kDa protein (Denker, Smith et al. 1988). Due to the localization of this protein in the RBCs, the brush border, and the nephron, it was suggested that it might be a water channel. The 28 kDa protein was then injected into oocytes bathed in a dilute solution to determine if it was capable of transporting water. The oocytes which had been injected with the protein swelled and exploded due to an excessive influx of water while there was no difference observed for the control oocytes (Preston, Carroll et al. 1992) (Figure 1).

There have now been several aquaporins (AQPs) identified in mammals. High resolution X-ray crystallography has been utilized to determine the structure of aquaporins. The aquaporin monomer is made up of two helical spanning domains and six helical domains going through the membrane, which helps transport H_2O across the hydrophobic region of the membrane (Verkman 2011). The channel itself consists of the assembly of four AQP monomers to form a tetramer. Through mutagenesis and molecular dynamic simulations it has been elucidated that some AQPs have a very narrow pore allowing transport of water

by single file transport. Others, known as aquaglyceroporins, have a less constricted pore and are capable of transporting not only water but also small solutes such as glycerol (Hub, Grubmuller et al. 2009, Khalili-Araghi, Gumbart et al. 2009). Despite a relatively low transport capacity, cells that express AQPs on plasma membranes have a markedly higher permeability to water (Verkman and Mitra 2000). This observed increase in permeability is due to the large abundance of AQPs seen on the membrane (Yang and Verkman 1997). AQPs have also been implicated in many other roles in the body other than water transport: cell migration, cell proliferation, and fat metabolism (Hara-Chikuma, Sohara et al. 2005, Hibuse, Maeda et al. 2005, Saadoun, Papadopoulos et al. 2005, Levin and Verkman 2006, Thiagarajah, Zhao et al. 2007).

Despite initial confusion as to how water is transported between cells and their extracellular environment, it is now known that cells have the capacity to transport water by diffusion as well as by facilitated diffusion through the use of proteins embedded in the membrane. One such group of membrane proteins, AQPs, have been shown to be specific for the transport of water molecules across the cellular membrane. The presence of membrane proteins with the primary role of water transport highlights the importance of water balance between compartments for overall function of the human body.

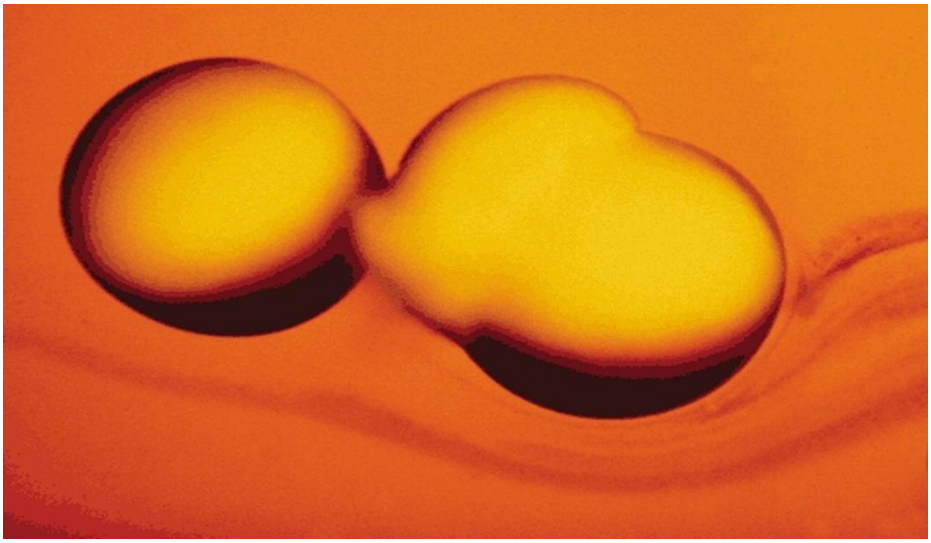


Figure 1. *Discovery of the first water channel. Incubation of control oocyte (Left) and oocyte injected with AQP1 cRNA (Right) in hypotonic solution. (Preston, Carroll et al. 1992)*

III. Hydration and Regulation of Fluid Shifts

The volume and composition of body fluids is primarily regulated by the renal system through the removal of both water and solutes. For cells to perform optimally it is necessary that they are in a stable internal environment. The balance between intake and output of both water and electrolytes is maintained primarily by the renal system. In brief, the renal system maintains this balance through adjusting the rates at which water and other solutes are secreted from and reabsorbed back into the blood plasma. There are two main forces that determine the rate at which plasma is filtered through the renal system: the hydrostatic pressure and the colloid osmotic pressure. Hydrostatic pressure in the capillaries tends to filter the plasma into the renal system, while the colloid osmotic pressure tends to draw the fluid into the capillaries due to plasma

proteins. Within the body there are several hormones which exert their effects on the renal system adjusting the secretion and reabsorption rate of water and particular solutes. Some of these hormones include atrial natriuretic peptide (ANP), antidiuretic hormone (ADH), and angiotensin II.

When there is an increase in the plasma volume, cells located in the cardiac atria are distended and as a result subsequently secrete ANP. (Maack, Camargo et al. 1985, Ballermann and Brenner 1986). This peptide directly acts on the nephron and inhibits the reabsorption of sodium and chloride primarily at the distal convoluted tubule (DCT) and collecting ducts (Zeidel 1990). The presence of ANP has also been shown to inhibit the secretion of renin and angiotensin II. Ultimately, the actions of ANP result in a decrease in plasma volume levels back towards resting values.

During periods of dehydration ADH will be released to increase the reabsorption of water. ADH is synthesized within the hypothalamus and then transported to the posterior pituitary for storage and eventual release through exocytosis (Douglas 1973, Hays 1976). Once released, ADH will travel through the plasma and bind to the basolateral surface of the collecting duct where it will increase the passive transport of water from the renal system back into the plasma at an increased rate of 7-50 fold (Hays and Leaf 1962, Morgan and Berliner 1968). This increase can be attributed to the translocation of AQP2 to the collecting duct to aid with the transport of water (Knepper and Inoue 1997).

The presence of angiotensin II increases during periods of decreased blood pressure or low extracellular volume. The formation of angiotensin II

occurs through the renin-angiotensin system (RAS). In brief, renin is released from the kidneys and cleaves angiotensinogen forming angiotensin I. After formation of angiotensin I it will then be converted by circulating angiotensin-converting enzyme (ACE) into the active angiotensin II. Angiotensin II will act on the nephron and increase the reabsorption of sodium, chloride, and water through the increase of basolateral Na^+/K^+ pump activity (Benigni, Cassis et al. 2010).

Hypernatremia and hyponatremia are two common clinical conditions associated with water imbalances. The normal serum concentration of sodium in the plasma ranges between 137-142 mEq/L. However, imbalances in water often cause a dysregulation in sodium balance (Ackerman 1990). When the concentration of sodium in the plasma exceeds 145mEq/L the disorder is known as hypernatremia. The causes of hypernatremia include excessive water loss and sodium gain, however; the most common cause tends to be loss of water. In some cases there may be defects in the osmoreceptors of the hypothalamus, which is known as adipsic hypernatremia. In these cases, the defect in the osmoreceptors lead to reduced osmoregulation by ADH resulting in decreased water reabsorption (Agrawal, Agarwal et al. 2008). The mortality rate of individuals with hypernatremia is 40%, but the incidence is less than 1% (Palevsky, Bhagrath et al. 1996). In contrast, hyponatremia occurs when the concentration of sodium in the plasma is less than 136mEq/L. The most common form of hyponatremia is hypotonic hyponatremia, however, isotonic hyponatremia also occurs. There are two mechanisms by which hypotonic hyponatremia occur:

1) normal water intake with impaired water excretion from the renal system (more common); and 2) water intake above normal levels that exceed the capacity of the renal system (Agrawal, Agarwal et al. 2008). Solute transport mechanisms (NKCC transporter, NaCl transporter, and ADH) of the nephron dictate diluting capacity, but dysfunction in these mechanisms lead to impaired water excretion as seen in hypotonic hyponatremia. Hyponatremia is much more common than hypernatremia in hospitalized patients (15-30%) and is associated with a 9-27% increase in mortality (Asadollahi, Beeching et al. 2006).

Regulation of whole body fluid composition and volume is largely performed through the actions of the renal system and associated hormones. These actions prevent any chronic dysregulations in water balance from occurring, however acute alterations still occur. These acute alterations can then subsequently impact cell volume and have large effects on cellular metabolism.

Chapter 1.2: Cell Volume Detection, Maintenance and Metabolism

I. Detection of Cell Volume

Following disturbances in cell volume, regulatory mechanisms are quickly activated but the mechanism by which these disturbances are detected is currently undetermined. Two main mechanisms have been proposed as to how cells detect changes in volume: 1) mechanical detection; and 2) osmotic detection (Parker 1993).

Ia. Mechanical Cell Volume Detection

The theory of mechanical cell volume detection refers to the belief that

cells send volume signals in response to mechanical disturbances such as bending, stretching, and rearrangement of internal structures. Since the postulation of this theory there has been very little supportive evidence, with more evidence refuting the theory. RBCs have been used extensively to study this theory due to their ability to swell to 1.6 times their normal volume before any stretching of the membrane occurs (Hoffman, Eden et al. 1958). Interestingly, it was observed that the regulatory volume mechanisms were activated when the cells were only 1.05-1.1 times their normal volume, before any cell stretching could occur (Parker 1993). This suggests that it is unlikely that these cells respond to membrane stretch when there is an increase in cell volume. Studies have also observed the response of RBCs to induced deformation (convex and concave) (Jennings and Schulz 1990). No alterations in KCl transport were observed in response to either deformation, leading to the conclusion that bending of the membrane is unlikely to be vital in detecting changes in cell volume. It has been shown that ghost cells (RBCs with similar composition but altered volume) with essentially only an ATP- regenerating system respond to swelling with an increase in the co-transport of KCl (Sachs 1988). However, during this study KCl transport was constitutively turned on so it is believed that the observed results are quite skewed (Parker 1993). The majority of evidence suggests that mechanical detection does not play a primary role in the detection of cell volume changes in RBCs. Therefore, future work should investigate the influence of mechanical changes on cell volume responses in a variety of cell types.

Ib. Osmotic Cell Volume Detection

Osmotic cell volume detection refers to the postulation that cells are capable of sensing alterations in volume through changes in the concentration of solutes and solvents (Sarkadi and Parker 1991). To determine the viability of this theory, several studies have investigated this hypothesis. It has been observed that ghost RBCs with reduced volume (33% of original), but identical surface area and hemoglobin concentration as intact RBCs, experience induced Na-H exchange (shrinking of cell) and induced KCl transport (swelling of cell) (Colclasure and Parker 1991, Colclasure and Parker 1992). The set point for volume in the ghosts was one third of that of the intact cells, but when the set point was plotted as a function of dry weight both cell types had the same set point. This finding suggests that it is not necessarily the volume of the cell, but rather the concentration of the solutes within the cell which is stimulating the cell volume regulatory mechanisms. In these studies, the set point of ghost cells containing ~100% hemoglobin was compared with cells containing 50% hemoglobin and 50% albumin and it was observed that they had the same set point, which represented that it was the concentration of protein in the cell that may be the monitored variable. Thiocyanate, urea, and other reagents have since been found to affect the volumes at which volume regulatory mechanism occur (Parker and Colclasure 1992, Parker 1993). It is believed that these specific reagents may impact the capacity of hemoglobin to detect alterations in macromolecular crowding or it may alter the susceptibility of some key enzymes to react to crowding.

Collectively, these results support the theory of macromolecular crowding, meaning that vital signalling reactions can be altered by small changes in intracellular protein concentrations (Minton 1983, Minton, Colclasure et al. 1992, Zimmerman and Minton 1993). More specifically, it supports the speculation that cells use macromolecular crowding as a means of detecting changes in cell volume (Zimmerman and Harrison 1987). From the current evidence, osmotic cell volume detection appears to be a more likely method of cell volume detection, however, it is possible that mechanical disturbances may play a secondary role.

II. Regulation of Cell Volume

Acute alterations in extracellular osmolality result in the transport of water into or out of cells. Once the changes in cell volume are detected, the volumes of the compartments need to be regulated. This involves the transport of osmolytes and the subsequent movement of water between intra- and extracellular compartments in an attempt to compensate for the osmotic induced shifts (Hoffmann, Lambert et al. 2009). These processes of cell volume regulation are known as regulatory volume increase (RVI) and regulatory volume decrease (RVD).

IIa. Regulatory Volume Increase (RVI)

After cell shrinkage, possibly due to exposure to a hyperosmotic extracellular environment, cells regain volume back towards resting levels through the process of regulatory volume increase (RVI). RVI is dependent on

the net influx of osmolytes and subsequent transport of water to increase cell volume. The movement of osmolytes into the cell is regulated through several transporters, which respond to alterations in cell volume. The main transport mechanisms involved in RVI include: Na^+/H^+ exchanger (NHE), NKCC cotransporter, taurine influx pathways, and hypertonicity-induced cation channels (HICCs). NHE functions to regulate the electroneutral import of one sodium ion in exchange for the removal of a hydrogen ion. This process will lead to a net influx and accumulation of sodium within the cell. The general consensus as to how NHE is activated by cell shrinkage is that there is an increase in the exchangers' affinity for H^+ allowing for it to be activated (Grinstein, Rothstein et al. 1985). Supporting this theory, it has also been observed that RBCs have a decreased affinity for Na^+ at an extracellular inhibitory site in response to cell shrinkage, thus activating the exchanger (Dunham, Kelley et al. 2004).

The NKCC cotransporter is responsible for the influx of Na^+, K^+, and Cl^- (1:1:2 respectively). It has been observed in several cell types that the NKCC cotransporter is phosphorylated at certain threonine residues on the NH_2 terminal end in response to cell shrinkage which will in turn activate the transporter (O'Donnell, Martinez et al. 1995, Lytle 1997, Gagnon, England et al. 2007).

Organic osmolytes are often used as a means of regulating cell volume over the long term. During RVI taurine has been shown to accumulate within the cell through taurine influx pathways. Taurine influx through this transporter is accompanied with Na^+ and Cl^-, and the stoichiometry has been estimated to be 1:2-3:1, respectively (Lambert 2004). Another contributing mechanism to RVI are

the HICCs, which have been shown to have a major role in some cell types, including; HeLa and glioma cells, during exposure to hyperosmotic stress (Wehner, Shimizu et al. 2003, Ross, Fuller et al. 2007, Plettenberg, Weiss et al. 2008). A summary of RVI related mechanisms can be found below (See Figure 2).

After cell shrinkage, it is necessary for cells to regain their volume towards resting values, a process known as RVI. This is accomplished through the actions of the Na^+/H^+ exchanger (NHE), NKCC cotransporter, taurine influx pathways, and hypertonicity-induced cation channels (HICCs). All of these transport pathways lead to the accumulation of osmolytes within the cell, ultimately leading to an increase in cellular volume by influx of H_2O. These mechanisms act quickly to prevent chronic decreases in cell volume, which can occur as a result of extracellular hyperosmotic stress.

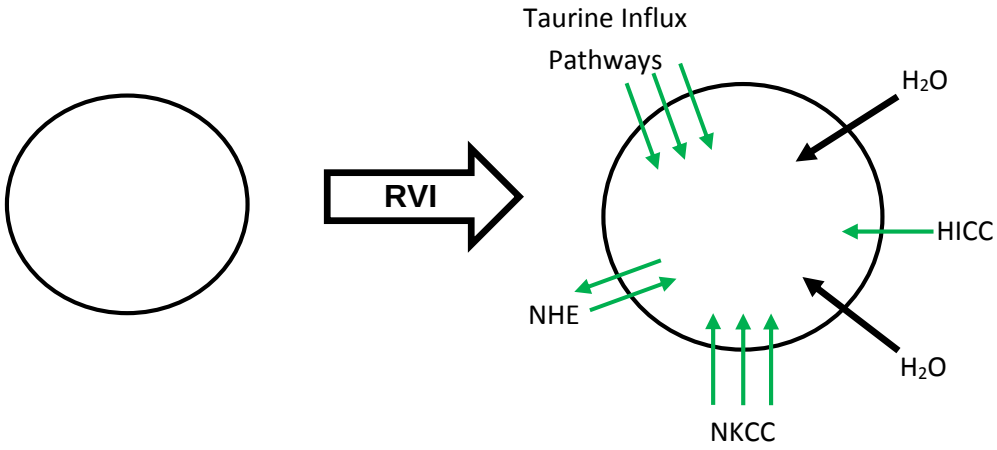


Figure 2. *Summary of regulatory volume increase (RVI) mechanisms. (Adapted from(Hoffmann, Lambert et al. 2009)). NHE-* Na^+/H^+ exchanger*, NKCC-* $Na^+/K^+/2Cl^-$ cotransporter*, HICC-* hypertonicity-induced cation channels

IIb. Regulatory Volume Decrease (RVD)

During acute exposure to a hypo-osmotic extracellular environment, cells exhibit a substantial increase in volume and must activate mechanisms in an attempt to export water to return back towards a more normal volume. This process of volume decrease is known as RVD and involves a net efflux of osmolytes from the cell as a means of extruding water. The membrane transporters that appear to regulate RVD are swelling activated Cl^- channels, swelling activated K^+ channels, K^+-Cl^- cotransporter (KCC), and taurine efflux pathways (Hoffmann, Lambert et al. 2009). The molecular identity of the swelling activated Cl^- channels are not yet known due to a lack of specific inhibitors, but they have been shown to be responsible for moving chloride out of the cell when cell volume is above normal (Nilius and Droogmans 2003). Several cell types (RBCs, CHO cells, endothelial cells) have shown that decreases in intracellular ionic strength, which will occur in response to hypo-osmotic stress, activate these channels and lead to the removal of chloride out of the cell (Cannon, Basavappa et al. 1998, Guizouarn and Motais 1999, Voets, Droogmans et al. 1999). It was also observed through single channel recordings that these channels are activated directly or through a voltage sensor in response to a reduction in intracellular osmotic stress. Similarly, there have also been a wide variety of K^+ channels identified that are volume sensitive and responsible for the efflux of K^+ out of the cell during RVD (Stutzin and Hoffmann 2006, Wehner 2006). Some potassium channels have been shown to be activated by membrane stretch.

However, in most cases it appears that activation is due to a combination of cell swelling and membrane stretch (Vanoye and Reuss 1999, Lesage and Lazdunski 2000). KCCs have been well documented as a mechanism of RVD in many cell types (Hebert, Mount et al. 2004, Gamba 2005). More specifically, it appears that the KCC1 isoform is expressed ubiquitously and its main physiological role is cell volume regulation. Several studies have observed that Ser/Thr phosphatase activity stimulates KCC activity while inhibition of this phosphatase reduces the activity (Jennings and Schulz 1991, Kaji and Tsukitani 1991, Bize, Munoz et al. 1998). The specific mechanism by which KCC is regulated by cell volume is currently unidentified. It is postulated to involve a volume-sensitive kinase that is inhibited and activated by swelling and shrinkage, respectively (Jennings and al-Rohil 1990). Another transporter that appears to play a role in RVD is the taurine efflux pathway. Similarly to the taurine influx pathway, the identity of the efflux pathway is not yet identified but it appears to be regulated by receptor tyrosine kinases (Lambert 2003, Franco, Lezama et al. 2004). A summary of RVD related mechanisms can be found below (See Figure 3).

After cell swelling, it is necessary for cells to decrease their volume towards resting values, a process known as RVD. This is accomplished through the actions of swelling activated Cl^- channels, swelling activated K^+ channels, K^+-Cl^- cotransporter (KCC), and taurine efflux pathways. All of these transport pathways lead to the extrusion of osmolytes out of the cell followed by H_2O, ultimately leading to a decrease in cellular volume. These mechanisms act quickly to prevent chronic increases in cell volume, which can occur as a result of

extracellular hypo-osmotic stress.

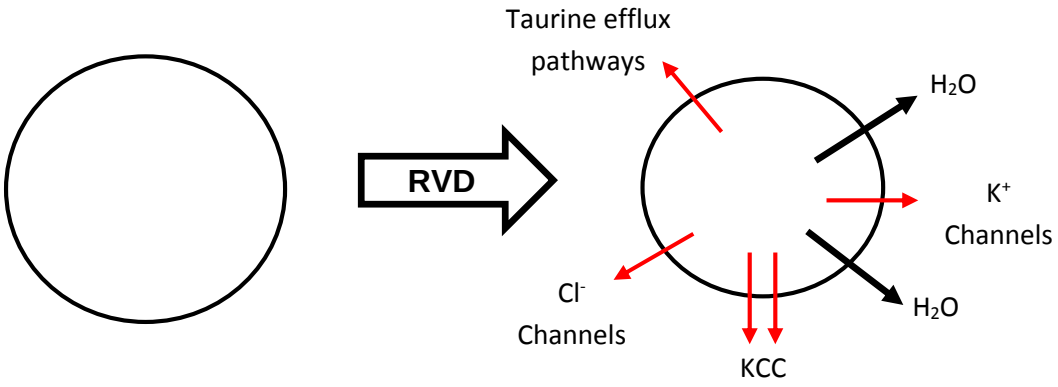


Figure 3. *Summary of regulatory volume decrease (RVD) mechanisms. (Adapted from(Hoffmann, Lambert et al. 2009)). KCC*- K^+-Cl^- cotransporter

III. Metabolic Implications of Cell Volume

Cell volume also appears to be an important signalling mechanism and has been shown to play an extensive role in regulating metabolism. Much of the work to date has been done in cell culture, using hepatocytes (Lang, Busch et al. 1998). In hepatocytes, exposure to insulin has been shown to cause increases in liver cell volume and subsequent alterations in metabolism. The cell swelling caused by insulin resulted in an increase in the synthesis of both proteins and glycogen while also inhibiting the degradation (Agius, Peak et al. 1994, Haussinger, Lang et al. 1994, Hussinger, Newsome et al. 1994). The addition of either glucagon or cAMP resulted in cell shrinkage leading to greater levels of both proteolysis and glycogenolysis, while also inhibiting protein synthesis. Several other hormones and factors have been shown to directly alter the volume

of hepatocytes and regulate their subsequent metabolism including ADH, ATP, H_2O_2, glucocorticoids, and amino acid uptake (Bakker-Grunwald 1983, Kuhlenschmidt, Hoffmann et al. 1991, vom Dahl, Hallbrucker et al. 1991, Hallbrucker, Ritter et al. 1993).

From the observations made in hepatocytes the “cell swelling theory” was postulated (Haussinger 1996). In general, this theory proposes that cell swelling creates an anabolic environment whereas shrinkage creates a catabolic one. The effects of alterations in cell volume on metabolism have been studied in hepatocytes, lymphocytes, fibroblasts, HeLa cells, and mammary cells (Robbins, Pederson et al. 1970, Clegg, Jackson et al. 1990, Wu and Flynn 1995, Millar, Barber et al. 1997). It has also been observed that in several clinical conditions that are characterized by hypercatabolism, the hypercatabolism was correlated with decreases in muscle cell volume (Haussinger, Roth et al. 1993).

More recently, the Roy lab has investigated the effects that osmotic stress have on carbohydrate metabolism in skeletal muscle. It was demonstrated that hyperosmotic stress induced cell shrinkage lead to an accumulation of lactate, glucose, and glucose-6-phosphate within both rat extensor digitorum longus (EDL) and soleus (SOL) (Antolic, Harrison et al. 2007, Farlinger, Harrison et al. 2007). They have also been able to demonstrate that metabolism of SOL and EDL are altered after contraction and exposure to an extracellular osmotic stress (Cermak, LeBlanc et al. 2009). More specifically, it was observed that after contraction, EDL experienced an increase in the active form of phosyphorylase (PHOS*a*) when exposed to hyperosmotic stress and a decrease in PHOS*a* when

exposed to hypo-osmotic stress. After contraction of SOL, an increase in pyruvate dehydrogenase a (PDH*a*) was observed during hyperosmotic stress. From these studies it is evident that more research is needed to better understand this area, especially in skeletal muscle.

Chapter 1.3: Skeletal Muscle and Protein Metabolism

Skeletal muscle contains ~75% of the intracellular fluid and ~33% of the interstitial fluid within the human body and is thus critical to whole body fluid balance (Hamilton, Ward et al. 1993). However, skeletal muscle is extremely susceptible to both chronic and acute fluctuations in cell volume. Skeletal muscle cells are large cells, with a significant capacity for plasticity in response to chronic resistance exercise or disuse, causing growth and atrophy, respectively (Taylor and Wilkinson 1986). They are also vulnerable to acute alterations in cell volume as a result of a single bout of exercise. It has been observed that in response to a maximal knee extension exercise in humans, intracellular muscle water content increased 15% and cell volume increased 15-20% (Sjogaard, Adams et al. 1985). Skeletal muscle also houses a large portion of the body's protein. Despite these characteristics, very little research has studied how changes in cell volume alter protein synthesis and breakdown in skeletal muscle.

<u>I. Protein Synthesis</u>

Protein synthesis is the process by which specific subunits known as amino acids combine to form long chains of polypeptides. One or more polypeptide chains combine and specific folding creates proteins with specific

functions. The sequence of amino acid residues is the primary determinant of overall function of a protein and this is highly regulated. The specific steps of protein synthesis include transcription followed by translation. In transcription, specific segments of DNA are used as a template to create a messenger RNA (mRNA) strand with complimentary nucleotides (except thymine is replaced with uracil). The formed mRNA acts as an intermediary transporting the genetic information of DNA to the protein synthesizing sites (ribosomes) (Brenner, Jacob et al. 1961, Jacob and Monod 1961). After transcription, the mRNA travels out of the nucleus and into the cytoplasm of the cell. From here, the ribosomes are capable of reading the mRNA strand and attaching the appropriate amino acids in sequence depending on the genetic code. Specific RNA molecules associated with the ribosome, known as transfer RNA (tRNA), are responsible for reading the genetic code of the mRNA and also bringing the specific amino acid to the ribosome (Chapeville, Lipmann et al. 1962, Grunberger, Weinstein et al. 1969). Through extensive research, it was determined that the tRNA was linearly reading groups of three nucleotides, known as codons, which are responsible for determining which of the twenty amino acids to add (Crick, Barnett et al. 1961). The rates of protein synthesis are altered in reponse to several factors such as exercise, disease, and hydration (Le Quesne, Spriggs et al. 2010, Churchward-Venne, Burd et al. 2012). The cell senses changes in the microenvironment which will in turn upregulate or downregulate signalling pathways, ultimately affecting cell activity and action. One of the most prominent signalling pathways involved in protein synthesis is the AKT/mTOR pathway.

Ia. AKT/mTOR pathway

The activation or inhibition of the Akt/mTOR pathway has been show to be critical to skeletal muscle hypertrophy and atrophy, respectively (Bodine, Stitt et al. 2001). The activation of this pathway is commonly initiated through the binding of growth factors to receptor tyrosine kinases (RTK) on the cell surface, however; energy status, oxygen levels, and amino acid availability have also been shown to regulate cell growth through this pathway (Laplante and Sabatini 2009). Binding of a substrate to the receptor causes internal dimerization and allows for the phosphorylation of specific tyrosine residues located intracellularly (Lemmon and Schlessinger 2010). Once the RTK is phosphorylated, phosphoinositide 3-kinase (PI3K) is then activated. Activation of PI3K will lead to the addition of a phosphate to the 3-OH group of phosphatidylinositol 4,5-bisphosphate (PIP2) forming phosphatidylinositiol 3,4,5-trisphosphate (PIP3) (Song, Ouyang et al. 2005). Levels of PIP3 within the body are highly regulated through phosphatases. One such phosphatase is the phosphatase and tensin homolog (PTEN), which acts to remove the phosphate from the 3-OH position on PIP3 reverting it back to the inactive PIP2 (Simpson and Parsons 2001). Formation of PIP3 will recruit phosphoinositide dependent kinase 1 (PDK1) and Akt to the cell membrane where it is located. Once translocated to the membrane, PDK1 will phosphorylate Akt at the Thr308 residue, subsequently activating it (Stephens, Anderson et al. 1998). Phosphorylation of Thr308 by PDK1 is said to only partially activate Akt, and to achieve full activation Ser473 must also be phosphorylated. The mechanism by which this occurs is

controversial (Alessi, Andjelkovic et al. 1996). After activation of Akt, it will indirectly activate mTOR through inhibition of the TSC1/TSC2 and also phosphorylation of proline rich Akt substrate of 40kDa (PRAS40) causing it to dissociate from mTOR (Manning, Tee et al. 2002, Wang, Harris et al. 2007). The energy status (ATP:ADP ratio) of the cell has also been shown to be regulate mTOR activation. More specifically, energy depletion will cause a decrease in the activation of mTOR, while periods of increased activation occur in response to an energy surplus (Laplante and Sabatini 2009)

The relationship between activation of mTOR and protein synthesis is well documented and it has been demonstrated that hyperactivation of the mTOR pathway promotes cell and tissue growth through elevated levels of protein synthesis (Sarbassov, Ali et al. 2005, Laplante and Sabatini 2009). The upregulation of protein synthesis is due to the subsequent activation of several downstream effectors which are altered as a result of mTOR activation (Laplante and Sabatini 2009). Downstream from mTOR are several effectors involved in protein synthesis which are active after phosphorylation through mTOR kinase activity. These downstream effectors include eukaryotic translation factor 4E-binding protein 1 (4E-BP1) and the p70 ribosomal S6 kinase 1 (p70S6K1) (Laplante and Sabatini 2009). Phosphorylation of 4E-BP1 results in an inability for it to bind to the eukaryotic initiation factor 4E (eIF4E), leaving eIF4E to promote cap-dependent translation (Richter and Sonenberg 2005). Activation of p70S6K1 will regulate several proteins and their respective activities: S6K1 aly-REF-like target (SKAR, mRNA biogenesis), programmed cell death 4 (PDCD4,

cap dependent translocation), eukaryotic elongation factor 2 kinase (eEF2K, translation elongation) and ribosomal protein S6 (ribosome biogenensis) (Ma and Blenis 2009). The regulation of all these proteins are vital to both cell survival and growth, and as such are highly regulated by the internal and external microenvironment. The Akt/mTOR pathway is shown in Figure 4.

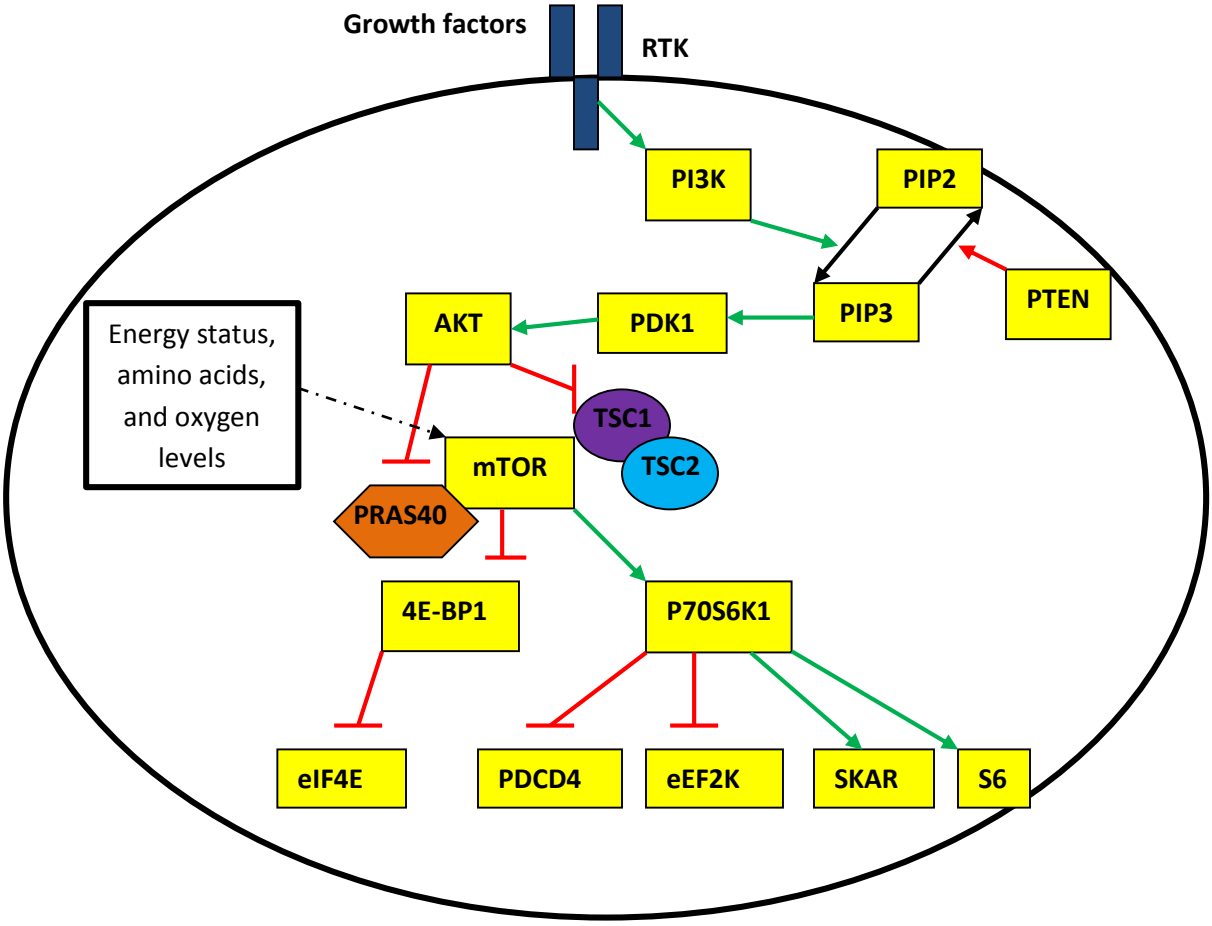


Figure 4. Depiction of the Akt/mTOR signalling pathway (Adapted from (Laplante and Sabatini 2009)). *RTK*- receptor tyrosine kinase. *PI3K*- phosphoinositide 3-kinase ,*PIP2*- phosphatidylinositol 4,5-bisphosphate, *PTEN*- phosphatase and tensin homolog, *PIP3*- phosphatidylinositiol 3,4,5-trisphosphate, *PDK1*- phosphoinositide dependent kinase 1, *AKT*- Protein Kinase B, *mTOR*- Mammalian target of rapamycin, *PRAS40*- proline rich Akt substrate of 40kDa, *P70S6K1*- p70 ribosomal S6 kinase 1, *4E-BP1* - eukaryotic translation factor 4E-binding protein 1, *eIF4E*- eukaryotic initiation factor 4E, *PDCD4*- programmed cell death 4, *eEF2K*- eukaryotic elongation factor 2 kinase, *SKAR*- S6K1 aly-REF-like target, *S6*- ribosomal protein S6

II. Protein Degradation

The other component of protein turnover is protein degradation; which is the process of breaking down proteins into their smaller subunits (amino acids, polypeptides) through the hydolysis of the peptide backbone. The degradation of proteins has many functions in the body including regulation of physiological and cellular processes, the removal of damaged and unwanted proteins, and the supply of amino acids for protein synthesis. Another role of protein degradation is the cleavage of several proteins known as proproteins which are synthesized in their inactive form and must be partially degraded to become active (Seidah and Chretien 1999). One such well known proprotein is proinsulin, partially digested into the active form of insulin (Winter, Lilie et al. 2002). Due to the dependency of the body on protein degradation for many vital functions, it is highly regulated by several systems. There are three major protein degradation pathways within muscle: the ubiquitin proteasome system, the calpain system, and the lysosomal system. (Dayton, Goll et al. 1976, Jagoe and Goldberg 2001, Bechet, Tassa et al. 2005). In mature muscle, it is thought that the ubiquitin proteasome and calpain systems contribute the greatest extent to muscle degredation in regards to cell volume due to the loss of lysosomal proteases as skeletal muscle ages. However, lysosomal activity has been shown to be stimulated in unison with the ubiquitin proteasome pathway and/or the calpain system in response to induced muscle wasting resulting from a wide array of states. These include cancer, muscle disuse, fasting, and head trauma (Wing and Goldberg 1993, Baracos,

DeVivo et al. 1995, Mansoor, Beaufrere et al. 1996, Taillandier, Aurousseau et al. 1996). Due to insufficient means of accurately isolating and measuring lysosomal proteolysis, the role that lysosomes may have on in vitro myofibrillar proteolysis in response to alterations in cell volume is currently unclear.

IIa. The Ubiquitin Proteasome System

The proteasome complex (26S) consists of a catalytic core particle (20S) and two regulatory cap sub-units (19S) (Walz, Erdmann et al. 1998). This system is responsible for the degradation of ubiquinated proteins. The tagging and activation process is initiated by the formation of a thio-ester bond between the molecule ubiquitin and a ubiquitin activating enzyme (E1) (Schulman and Harper 2009). After activation, ubiquitin is then transferred from the E1 to a ubiquitin conjugating enzyme (E2) where it will form a high energy conjugate by means of a catalytic cysteine residue (van Wijk and Timmers 2010). From here, the E2 with an attached ubiquitin will interact with specific ubiquitin ligases (E3). E3s have the capacity to bind to specific substrates (proteins). The interaction between E2 and E3 will result in the transfer of ubiquitin to the protein, resulting in the protein becoming tagged. For each protein to be degraded, this sequence will occur four times at minimum. This is due to the proteasomal lid recognizing ubiquitin chains of only four or greater (Thrower, Hoffman et al. 2000). After polyubiquination, the protein substrate will be detected by the proteasome and ultimately degraded leaving the ubiquitin molecules to be recycled for later use (Figure 5).

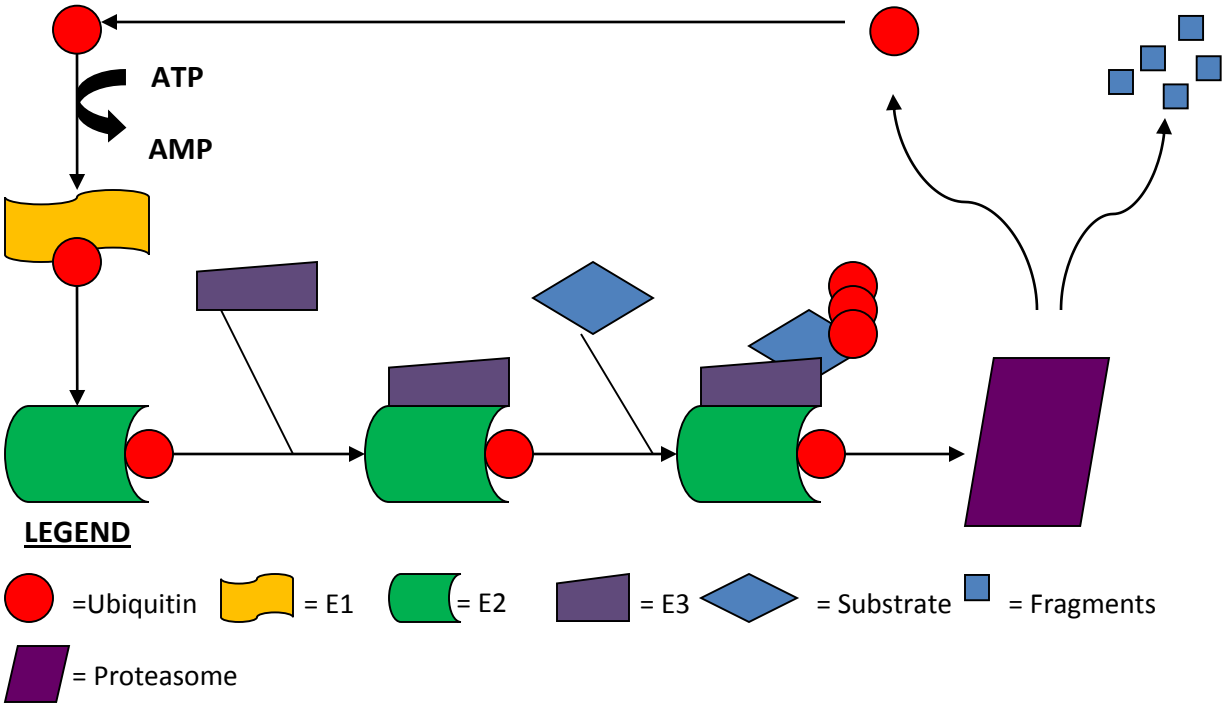


Figure 5. *Depiction of the ubiquitin proteasome pathway (Adapted from (Rahimi 2012)). E1*- ubiquitin activating enzyme, *E2*- ubiquitin conjugating enzyme, *E3*- ubiquitin ligase

There are a wide variety of E3s and only a few have been observed to be upregulated during periods of muscle atrophy (Sacheck, Hyatt et al. 2007). The comparison of gene expression in different models of muscle atrophy led to the discovery of commonly regulated genes that are linked to the loss of muscle components, known as atrophy-related genes or atrogenes. More specifically, it was observed that two genes were expressed more strongly than others and encoded the muscle specific E3s atrogin-1 (MAFbx) and muscle RING-finger

protein-1 (MuRF1) (Bodine, Latres et al. 2001, Gomes, Lecker et al. 2001). The knockdown of atrogin-1 in mice has been shown to prevent atrophy during fasting, while MuRF1 knockout mice are resistant to dexamethasone induced muscle loss (Baehr, Furlow et al. 2011, Cong, Sun et al. 2011). Other ubiquitin ligases have been identified to be upregulated during muscle atrophy, but they appear to play a much smaller role than either atrogin-1 and/or MuRF1. Some of these ligases include Trim32, TRAF6 and CHIP (Arndt, Dick et al. 2010, Paul, Gupta et al. 2010, Cohen, Zhai et al. 2012).

IIb. The Calpain System

Another proteolytic system which has a large role in the degradation of myofibrillar proteins is the calpain system. Calpains are calcium activated cysteine proteases responsible for nonspecific proteolysis. Calcium, phospholipids and calpastain (a specific calpain inhibitor) have been identfied to play a role in the regulation of calpain activity (Kawasaki, Emori et al. 1993, Saido, Sorimachi et al. 1994). They are found ubiquitously in mammals with a number of tissue specific isoforms also identified (Sorimachi, Saido et al. 1994). Two main forms of calpains have been identified in mammals, the m- and µ-calpains. The main difference between the two forms is their calcium sensitivities. m-calpain requires millimolar Ca^{2+} concentrations to be activated, whereas µ-calpain only requires micromolar concentrations for activation (Suzuki, Hata et al. 2004). These forms of calpain are found as heterodimers, consisting of an 80kDa catalytic subunit and a 30kDa regulatory unit.The regulatory units are similar between forms of calpain whereas the catalytic subunits differ.

A general mechanism of calpain activation has been proposed where calpain translocates from the cytosol to the membrane in response to Ca^{2+}. After translocation to the membrane it is further activated by calcium or phospholipids. This activation causes autocatalytic hydrolysis which results in the dissociation of the 30 kDa regulatory unit from the 80kDa catalytic unit leaving the catalytic domain free to hydrolyze proteins at both the membrane and in the cytosol (Suzuki, Sorimachi et al. 1995). More specifically, it is postulated that the binding of calcium to one of several binding sites on calpain leads to the rearrangement of two subdomains bringing them closer together and creating a functional catalytic site and dissociation of the 80kDa and 30kDa units (Hosfield, Elce et al. 1999, Moldoveanu, Hosfield et al. 2002). When calcium is not present in the necessary concentrations the two subdomains remain separated despite being entropically unfavourable due to the stability contributions from formed salt bridges between amino acid residues (Suzuki, Hata et al. 2004).

Resting levels of Ca^{2+} in mammalian cells are maintained at approximately 50nM (Berchtold, Brinkmeier et al. 2000). This concentration of calcium is significantly less than that required to activate either m- or μ-calpain (millimolar and micromolar respectively). Elevated levels of calcium are necessary for many physical functions and signalling, such as skeletal muscle contraction, but could also activate myofibrillar degradation by the calpain system. In skeletal muscle, calcium levels are elevated in response to several disorders and environmental conditions. There are chronically elevated levels of calcium in individuals with Duchenne muscular dystrophy, and acute increases are seen in response to

osmotic stress (Turner, Fong et al. 1991, Martin, Petousi et al. 2003). More specifically, hyperosmotic stress has been shown to increase free calcium levels within the cytosol while calcium appears to be unaffected in hypo-osmotic conditions. These increases in calcium with extracellular hyperosmotic stress have been termed “calcium sparks” (Cheng, Lederer et al. 1993). Calcium sparks are acute brief increases in free calcium within the cytosol which occur without the presence of a muscle action potential. The exact mechanism by which calcium sparks occur in response to extracellular osmotic stress is not completely known. It has been postulated that it could be due to changes in the triadic junction, deformation of the membrane, and/or increased reactive oxygen species (ROS) (Isaeva, Shkryl et al. 2005, Wang, Weisleder et al. 2005, Teichmann, Wegner et al. 2008). It was recently suggested that hyperosmotic stress causes changes in the membrane potential altering the calcium storage and transport systems of the muscle. It is believed that the significant decrease in muscle cell volume caused by extracellular hyperosmotic stress causes an alteration in how the ryanodine receptors (RR) and dihydropyridine receptors (DHPR) interact with each other, resulting in the release of calcium from the SR into the muscle cell (Apostol, Ursu et al. 2009). Supporting this theory, it was observed that inhibiting DHPR also inhibited the associated increase in calcium seen previously when exposed to an extracellular hyperosmotic stress (Pickering, White et al. 2009). Despite not yet knowing the mechanism underlying the increase in intracellular free calcium, it is evident that these increases do in fact occur, and that calpain could be activated in response to such stress

(Darling, Vandommele et al. 2011).

III. Protein Turnover and Osmotic Stress in Skeletal Muscle

As previously mentioned, most research regarding osmotic stress and metabolism has been conducted in hepatocytes. Recently, several lab groups have taken it upon themselves to increase the foundation of knowledge concerning the implication of osmotic stress on the protein turnover in skeletal muscle. These studies have employed both cell culture and animal models.

In 2009, our lab conducted a study which is believed to be the first to demonstrate that acute alterations in osmolarity have large effects on protein metabolism in skeletal muscle cells (Roy, Russell et al. 2009). This study used differentiated L6 myoblasts that were placed into one of the three extracellular osmotic conditions (Control, Hypo-osmotic, or Hyperosmotic) and incubated for either 24 or 48 hours. During the last hour of incubations, ^{3}H labeled tyrosine incorporation was assessed and used as a measure of protein synthesis. It was observed that protein synthesis was supressed within the cells that were exposed to hyperosmotic stress but there was no change seen for the hypo-osmotic stress (Figure 6).

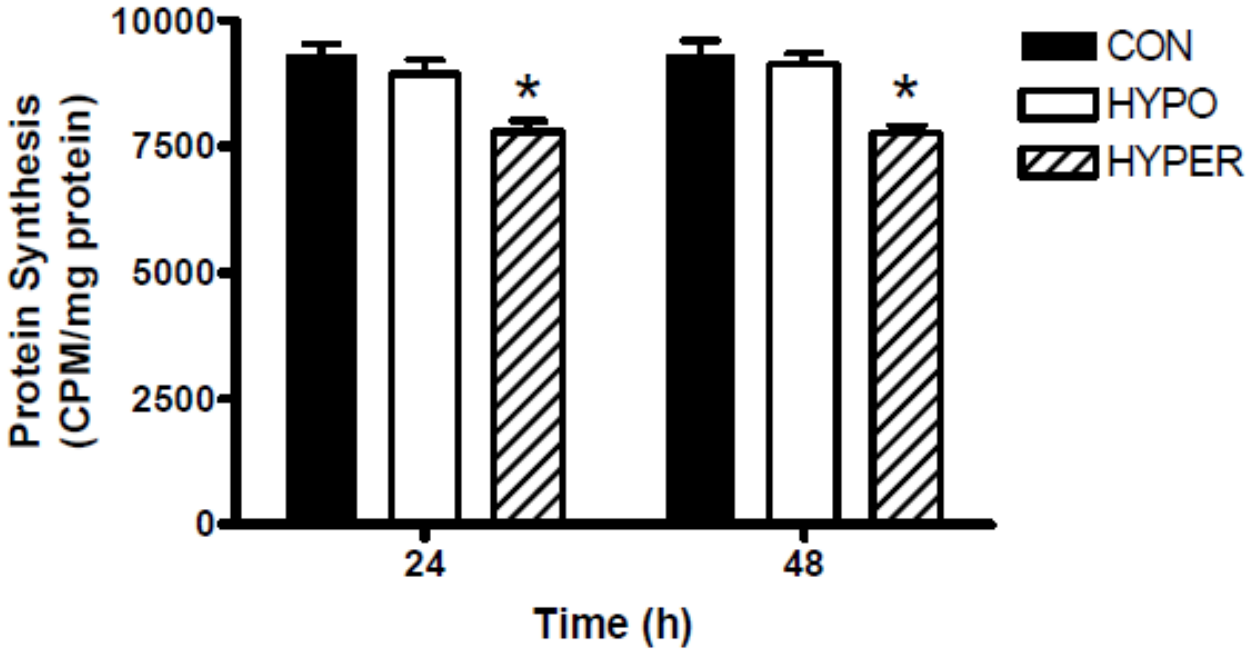


Figure 6. *Protein synthesis rates as measured by incorporation of radioactive tyrosine during final hour of incubation in L6 cells. * denotes a significant difference from CON condition (*p<0.05). CON*- Control, *HYPO*- Hypo-osmotic stress, *HYPER*- Hyperosmotic stress. (Roy, Russell et al. 2009)

More recently, studies incorporating the analysis of protein synthesis along with markers of protein degradation were conducted using isolated skeletal muscle from male Long-Evans rats (Darling, Vandommele et al. 2011). This model allows for acute rapid and reversible alterations in cell volume due to extracellular osmotic conditions (Antolic, Harrison et al. 2007, Cermak, LeBlanc et al. 2009). In brief, rat extensor digitorum longus (EDL) muscles were incubated in either hyperosmotic (HYPER), hypo-osmotic (HYPO), or iso-osmotic (ISO) media for 135 minutes. During the final 75 minutes ^{14}C labeled phenylalanine or cyclohexamide were added to the bathing media. After the prescribed incubation

time, phenylalanine uptake and incorporation in the muscle protein were measured along with the appearance of tyrosine in the media. The results showed that HYPO lead to significant increases in protein synthesis when compared to ISO and significant decreases in HYPER compared to ISO (Figure 7).

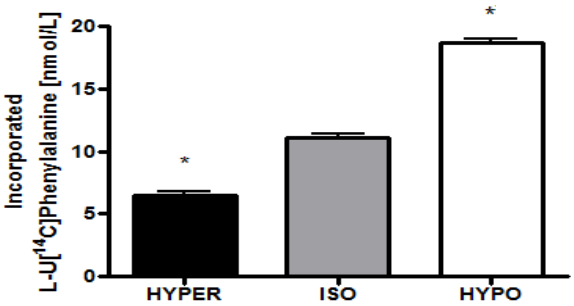


Figure 7. *Protein synthesis rates as measured by incorporation of radioactive phenylalanine during final hour of incubation in rat skeletal muscle($p<0.05$). * denotes a significant difference from ISO condition*. *ISO*- Iso-osmotic, *HYPO*- Hypo-osmotic stress, *HYPER*- Hyperosmotic stress. (Darling, Vandommele et al. 2011).

As for protein degradation, it was observed that HYPER led to significantly greater rates of protein degradation than ISO, however; there was no noticeable differences between HYPO and ISO (See Figure 8). Protein quantification was also conducted after exposure to the experimental conditions, and western blots demonstrated that mTOR phosphorylation at Ser2448 was elevated in both

HYPER and HYPO when compared to ISO, while phosphorylation of p70s6k was increased in HYPO and decreased in HYPER when compared to ISO.

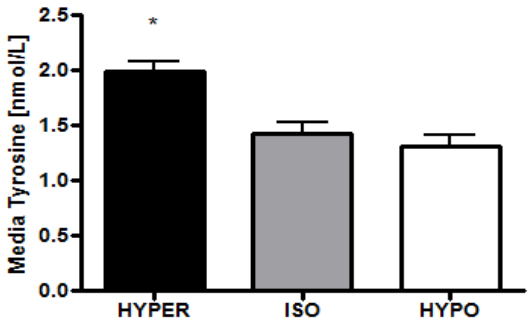


Figure 8. *Protein degradation rates measured as appearance of tyrosine in the media following incubation in rat skeletal muscle($p<0.05$). * signifies a significant difference from ISO condition. ISO*- Iso-osmotic, *HYPO*- Hypo-osmotic stress, *HYPER*- Hyperosmotic stress. (Darling, Vandommele et al. 2011).

From the available findings it appears that acute hyperosmotic stress creates an intracellular catabolic environment whereas acute hypo-osmotic stress creates an anabolic environment within skeletal muscle. However, this has only been observed during acute periods of exposure at a limited number of timepoints. What remains unclear is the time course response in protein synthesis and degradation to extracellular hyper and hypo-osmotic stress within skeletal muscle. To fully understand the influence of extracellular osmotic stress on protein metabolism in skeletal muscle it is necessary to observe the impact at multiple points along a timeline using a consistent model.

Chapter 2: Statement of the Problem

I. Statement of the Problem

To date there has been limited research on the influence of extracellular osmotic stress on protein turnover in skeletal muscle, and this work has only investigated a limited time frame of protein metabolic responses. In L6 skeletal muscle cells specifically, only incubation periods of 24 and 48 hours have been investigated and these only looked at protein synthesis and not degradation. In whole muscle tissue, incubation periods of 135 minutes in hyperosmotic stress resulted in a negative protein balance and hypo-osmotic stress led to a positive protein balance, however what remains unclear is the actual time course of how muscle responds to extracellular hyper and hypo-osmotic stress.

II. Purpose

The purpose of this thesis is to:

1) Establish the time dependent influence of both extracellular hyperosmotic and hypo-osmotic stress on protein synthesis in L6 skeletal muscle cells.

2) Determine the influence of extracellular osmotic stress (hyperosmotic and hypo-osmotic) on the ubiquitin proteasome and calpain protein degradation pathways during the established time course.

3) Determine the influence of extracellular osmotic stress (hyperosmotic and hypo-osmotic) on the Akt/mTOR/p70s6k protein synthesis signalling pathway through observation of the phosphorylation state of mTOR and p70s6k during the established time course.

III. Hypotheses

In regards to the aforementioned purpose, it is hypothesized that:

1) Hyperosmotic stress will induce decreased rates of protein synthesis in L6 skeletal muscle cells, whereas hypo-osmotic stress will induce elevated levels of protein synthesis. It is expected that the alterations in protein synthesis will attenuate as the duration of incubation increases.

2) Hyperosmotic stress will cause an increase in proteasomal, and calpain activity represented as an increase in the amount of polyubiquitinated proteins and autolysis of μ-calpain. While hypo-osmotic stress will decrease levels of proteasomal, and calpain activity represented as a decrease in the amount of polyubiquitinated proteins and autolysis of μ-calpain

3) Hyperosmotic stress will cause a down regulation of the Akt/mTOR/p70s6k protein synthesis signalling pathway, represented as a decrease in the phosphorylation of both mTOR and p70s6k. While hypo-osmotic stress will cause an up regulation of the Akt/mTOR/p70s6k protein synthesis signalling pathway, represented as an increase in the phosphorylation of both mTOR and p70s6k. (See Figure 9 for a summary of hypotheses)

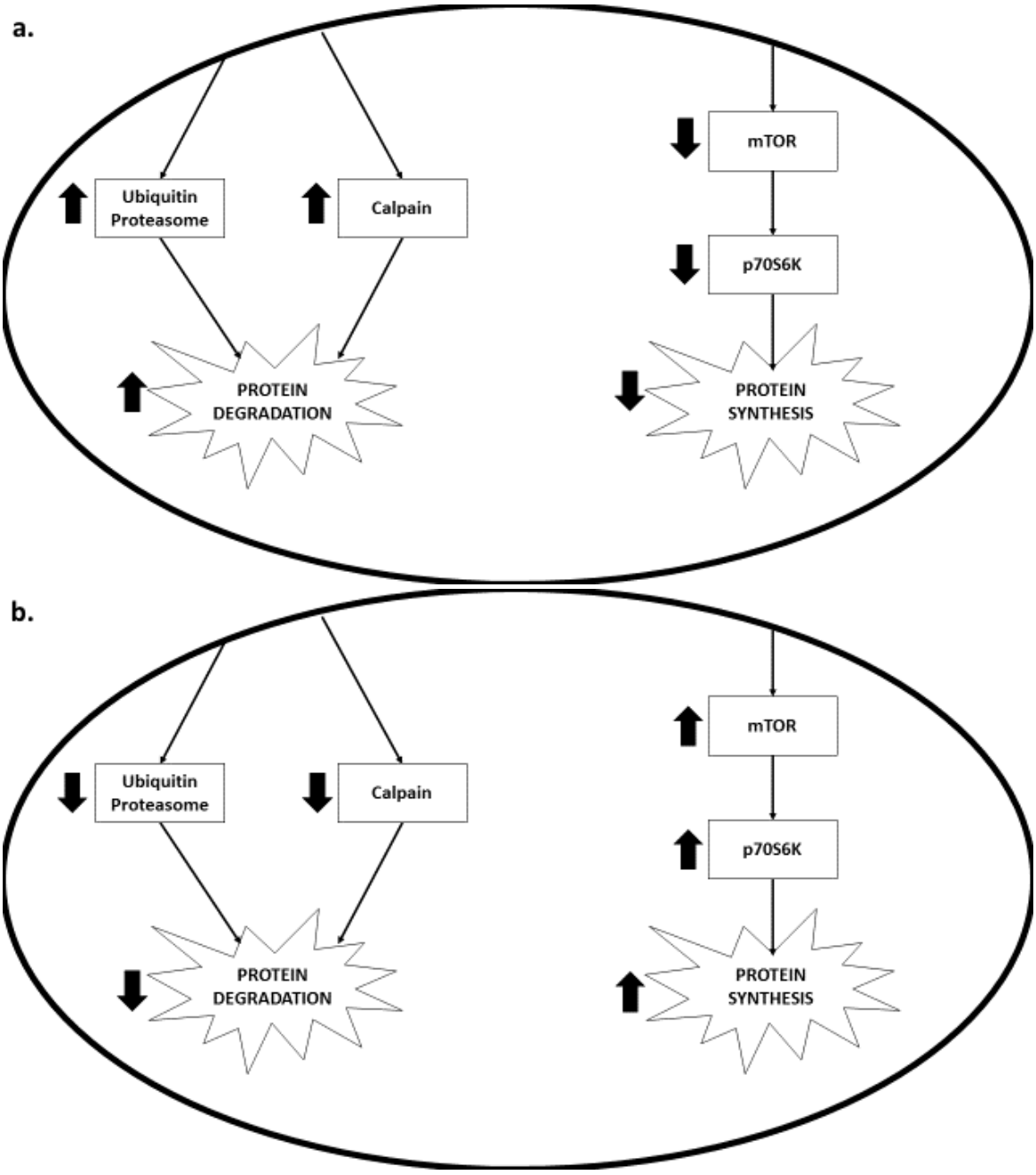


Figure 9. *Visual representation of hypothesized outcomes in L6 muscle cells in response to; a) hyperosmotic stress, b) hypo-osmotic stress. mTOR = mammalian target of rapamycin, p70S6K = p70 Ribosomal S6 Kinase.*

Chapter 3: Methodology

I.Cell Culture

Undifferentiated L6 myoblasts (American Type Culture Collection, Manassas, VA) were plated on T75cm^2 cell culture flasks (Diamed, Mississauga, ON) and proliferated in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Burlington, ON) and 1% penicillin-streptomycin (Sigma-Aldrich, Oakville, ON) until ~80% confluency was reached (~3 days). While culturing, all cells were incubated at 37°C in 5% CO_2 and media was changed every other day. After 80% confluency was reached, cells were counted and viability was assessed through the extrusion of Trypan blue observed through an inverted microscope (Primovert; Zeiss, Toronto, ON) (Louis and Siegel 2011). Myoblasts (passage 7) were plated on multiwell 6 well plates at a density of 100000 cells/well (Diamed, Missisauga, ON) and allowed to proliferate for 24 hours. Following 24 hours of proliferation, differentiation was induced by the substitution of 10% fetal bovine serum with 2% adult horse serum (AHS; Life Technologies, Burlington, ON) in the media. L6 myoblasts were differentiated for ~7 days.

II. Osmotic Conditions

Three experimental osmotic conditions were used during incubations: hyper-osmotic, hypo-osmotic, and a control. All three consisted of DMEM containing 2% AHS, and 1% penicillin-streptomycin; which served as the control

condition. D-mannitol (Sigma-Aldrich, Oakville, ON) was added (~17mg/ml) to media to induce a hyper-osmotic stress; while media was diluted with distilled water (1ml dH_2O/ 2.5ml media) to induce a hypo-osmotic stress. The pH of the media was then adjusted to 7.4, and osmolality was measured using a vapour pressure osmometer (VAPRO5520; Wescor, Logan, UT).

III. Measurement of Protein Synthesis

Protein synthesis was assessed through incorporation of trichloroacetic acid (TCA) insoluble radioactivity (Gulve and Dice 1989). In brief, L6 myoblasts were incubated in one of the three osmotic conditions for 4, 8, 12, or 24 hours (2ml/well) (Figure 10). During the final 4 hours of incubation, media was supplemented with 1.0µCi of L-[ring-3,5-^{3}H]-tyrosine/ml (Perkin Elmer, Woodbridge, ON) and 2mM of L-tyrosine (AMRESCO, Solon, OH). Immediately following incorporation of radiolabeled tyrosine, media was discarded and cells were washed three times with cold (4°C) phosphate buffered saline (PBS; Life Technologies, Burlington, ON) to remove any excess radioisotope. Cold 10% TCA (2ml, 4°C) was then added to each well and incubated on ice for ~10min. Following the incubation, the cell layer in TCA was scraped off and transferred to a 2ml eppendorf and incubated on ice for 1 hour. Eppendorfs were then centrifuged at 20000x g for 10 min at 4°C in an Allegra 21R centrifuge (Beckman Coulter, Mississauga, ON). Following initial centrifugation, the pellet was washed with 1ml 10% TCA (4°C) and centrifuged again at 20000x g for 10 min at 4°C. After centrifugation, the supernatant was removed and 0.5ml of 0.1 M NaOH/1%

Triton X-100 was added to the pellet overnight to dissolve the protein precipitate into solution. Once dissolved, the sample was vortexed and 0.4ml was added to 4ml of scintillation fluid (Scintisafe Econo1; Fisher Scientific, Ottawa, ON) for determination of TCA-insoluble radioactivity by scintillation counting ((LS6500; Beckman Coulter, Mississauga, ON) Maximum ^{3}H count rate = 10 x 10^{6} CPM, estimated ^{3}H counting efficiency = ~65%). A tritiated standard (4mL scintillation fluid, 0.4mL 0.1 M NaOH/1% Triton X-100, and 0.5µCi of L-[ring-3,5-^{3}H]-tyrosine) was concurrently assessed with samples to verify scintillation counter function. Protein concentration of the sample was determined through a Bradford assay, using 80µl of the remaining sample with 400µl of dH_2O (Harlow and Lane 2006). To calculate protein synthesis, myotube radioactivity was normalized for protein concentration using the equation below (Eq. 1).

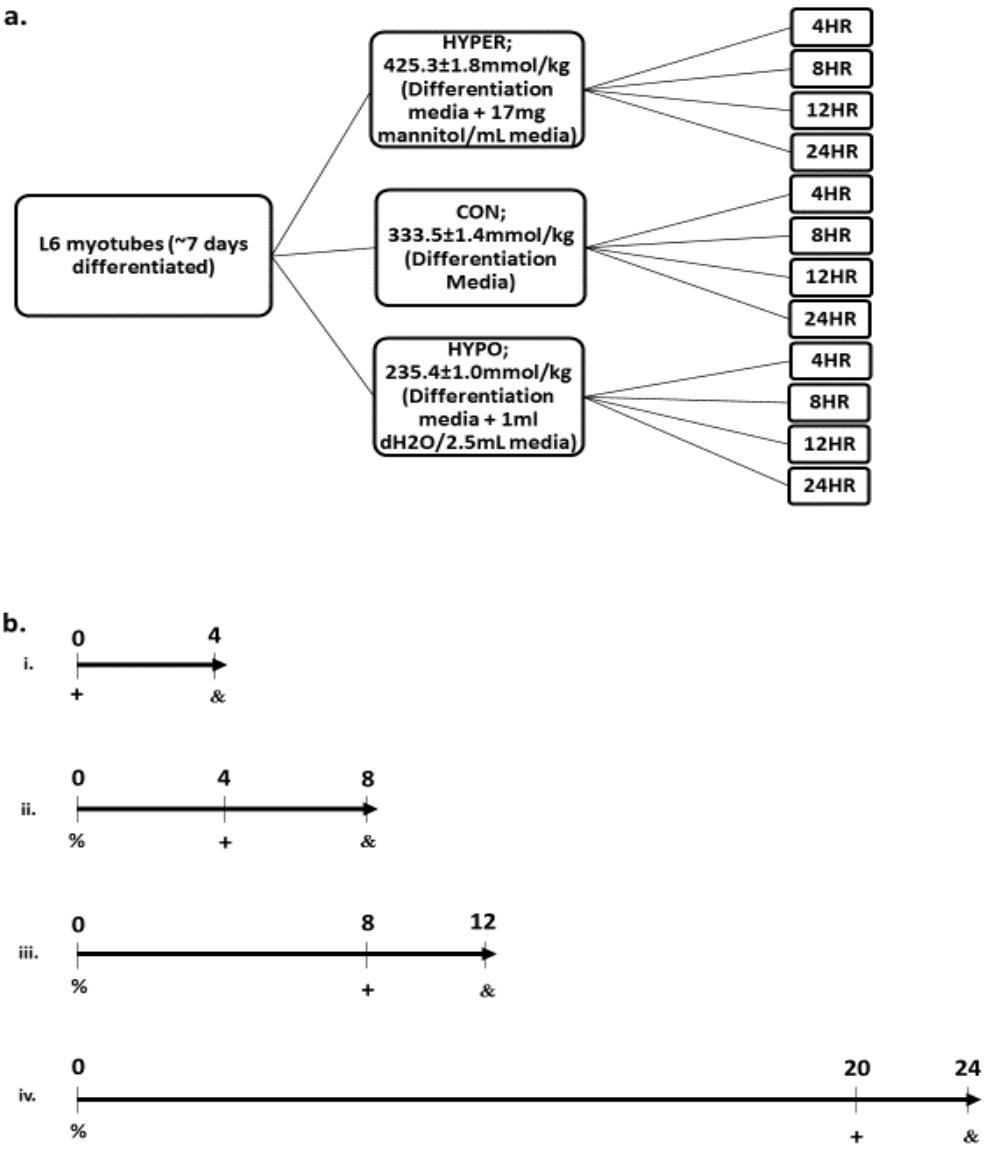


Figure 10. *a) Experimental design. HYPER= Hyperosmotic stress, CON= Control, HYPO= Hypo-osmotic stress. b) Experimental timelines; time (hours) is found above each line while the symbols below represent specific actions; i) 4 hours of stress, ii) 8 hours of stress. iii) 12 hours of stress, iv) 24 hours of stress.* ***%*** *= incubation in osmotic condition,* ***+*** *= addition of 1.0µCi of L-[ring-3,5-^{3}H]-tyrosine/ml,* ***&*** *= removal of media and measurement of protein synthesis or extraction of protein.*

Eq. 1 $$Protein\ Synthesis = \frac{MR(cpm)}{mg\ protein}$$

Where MR= Myotube protein radioactivity (measured in cpm-counts/minute)

IV. Western Blotting Analysis

Western blots were performed to assess the relative content of several proteins involved in protein synthesis and degradation signalling pathways. More specifically, the proportion of phosphorylated mTOR and p70S6K1 were compared to total mTOR and p70S6K1 respectively to assess protein synthetic markers; and the autolysis of calpain and polyubiquitination of proteins (normalized to actin) were used to assess protein degradation markers. This was determined through the use of specific antibodies for the proteins of interest and their phosphorylated states; rabbit polyclonal mTOR (2972S; Cell Signalling, Danvers, MA), rabbit monoclonal phospho-mTOR (Ser 2448) (2971S; Cell Signalling, Danvers, MA), rabbit polyclonal p70S6K α (sc-230; Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal phospho-p70S6K α (Thr 389) (sc-11759; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal µ-Calpain (2556; Cell Signalling, Danvers, MA), mouse monoclonal Ub (P4D1) (sc-8017; Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal actin (I-19) (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA).

Following incubation in the experimental media for the prescribed time, cells were washed twice with ice cold PBS (4°C). RIPA buffer (ab156034; abcam, Toronto, ON) containing phosphatase inhibitors (PhosSTOP; Roche, Laval, QC)

and protease inhibitors (cOmplete; Roche, Laval, QC) was added (0.1ml/well) and cells were subsequently scraped on ice and transferred to a 1.7ml eppendorf. The eppendorfs were then vortexed for ~1 min and placed on a lab rotator (Thermo Scientific, Waltham, MA) for 1 hour at 4°C. Following this, the eppendorfs were spun at 15000x g for 15 min at 4°C and the resulting supernatant was transferred to a new eppendorf.

To determine total protein content a Bradford assay was conducted on a fraction of the cell lysate. The cell lysate was diluted accordingly with the appropriate amounts of dH_2O and sample buffer (0.5M Tris-HCl, 2mL glycerol, 10% SDS, 1% bromophenol blue, 1 mL β-mercaptoethanol, and dH_2O) to a protein concentration of 1µg/µL. Running (7.5% for phospho-mTOR/mTOR/µ-calpain/actin/Ub, and 10% for phospho-p70S6K1 α/p70S6K1 α) and stacking (4% for all samples) gels were made and standard SDS electrophoresis was performed. 5µg of protein was loaded per well to assess phospho-mTOR/mTOR, while 10µg of protein was loaded per well to assess phospho-p70S6K1 α/p70S6K1 α/ µ-calpain/actin/Ub. Samples were then electrophoretically ran in running buffer (495ml dH_2O, 5ml 10% SDS, 250mM TRIS, and 1.92 mM glycine) along with a protein standard (Precision plus protein kaleidoscope; BioRad, Mississauga, ON) to separate proteins (See appendix for specific times and voltages). Once samples were allowed to run for the appropriate amount of time, proteins were transferred in transfer buffer (800ml dH_2O, 200 ml methanol, 31.25mM TRIS, and 240mM glycine) on to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was then blocked using TBST (20mM tris

base, 137mM NaCl, and 0.1% tween 20, pH 7.5)-milk (2.5% for 1 hour- p70S6K1 α, 5% for 1 hour- phospho-p70S6K1 α/mTOR/phospho-mTOR/μ-calpain/actin/Ub). Blocking solution was discarded, and PVDF membranes were washed 4x for 5 minutes in TBST. Membranes were then incubated overnight at 4°C in 5% TBST-milk (for p70S6K1 α/Ub/actin) or 5% BSA-TBST (for mTOR/phospho-mTOR/phospho- p70S6K1 α/μ-calpain) containing specific 1° antibodies at a 1:1000 dilution (previously mentioned). Following overnight incubation, membranes were once again washed 4x for 5 minutes in TBST. After washing, membranes were incubated for 1hr with an appropriate 2° antibody diluted in either 5%milk-TBST or 5%BSA-TBST (See appendix for specific antibodies and dilutions). Membranes were washed 4x for 5 minutes, and Immobilon western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore, Etobicoke, ON) was applied directly to the membrane for 5 min. Membranes were then developed through enhanced chemilumiscence using the Fluorchem5500 imaging system (Alpha-Innotec, Santa Clara, CA) and AlphaEaseFC software (Alpha-Innotec, Santa Clara, CA). Images were analysed using Image-J software (National Institutes of Health, Bethesda, MD).

VII. Statistical Analysis

Values are expressed as mean ± the standard error of the mean (SEM). Statistical significance was evaluated through the use of a two way ANOVA evaluating the factors of time (4, 8, 12, or 24 hours) and osmolality (HYPER, CON, or HYPO) using the GraphPad Prism™ v5 (GraphPad, La Jolla, CA)

statistical program. A Bonferroni post hoc test was conducted if a statistically significant interaction was detected. Results were considered statistically significant when $p<0.05$.

Chapter 4: Results

I. Osmolality of Experimental Media

Prior to incubation and by design, the media osmolality of all groups were found to be significantly different from one another. HYPER (425.3 ± 1.8mmol/kg) was found to have the greatest osmolality, followed by CON (333.5 ± 1.4mmol/kg) and then HYPO (235.4 ± 1.0mmol/kg) HYPO (Figure 11).

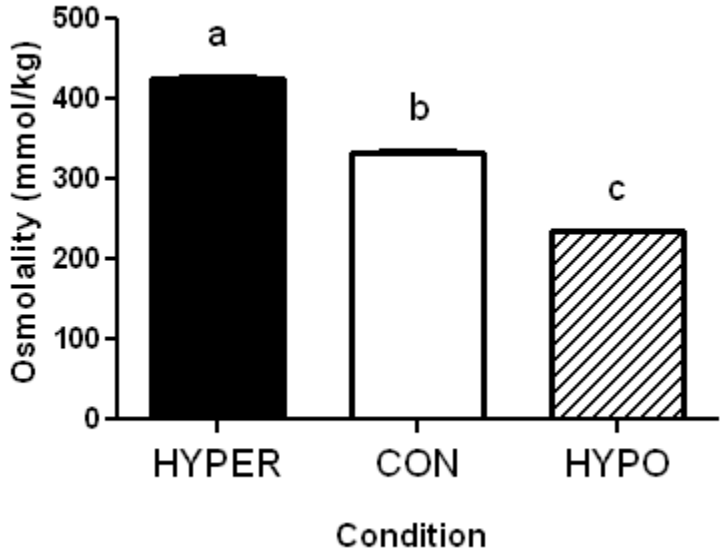


Figure 11. *Measured osmolality of experimental media prior to incubation. Values expressed as mean ± SEM (n=12/group). Means with different letters are significantly different (p<0.05). HYPER=Hyperosmotic stress, CON=Control, HYPO=Hypo-osmotic stress.*

II. Protein Synthesis

Following a 4 hour incubation in media supplemented with L-[ring-3, 5-^{3}H]-tyrosine, cells were harvested and protein was assessed for relative myotube radioactivity through scintillation counting. The resulting measurements did not

differ between any of the conditions. As such, there was no significant interaction or main effects for time or osmolality (Figure 12).

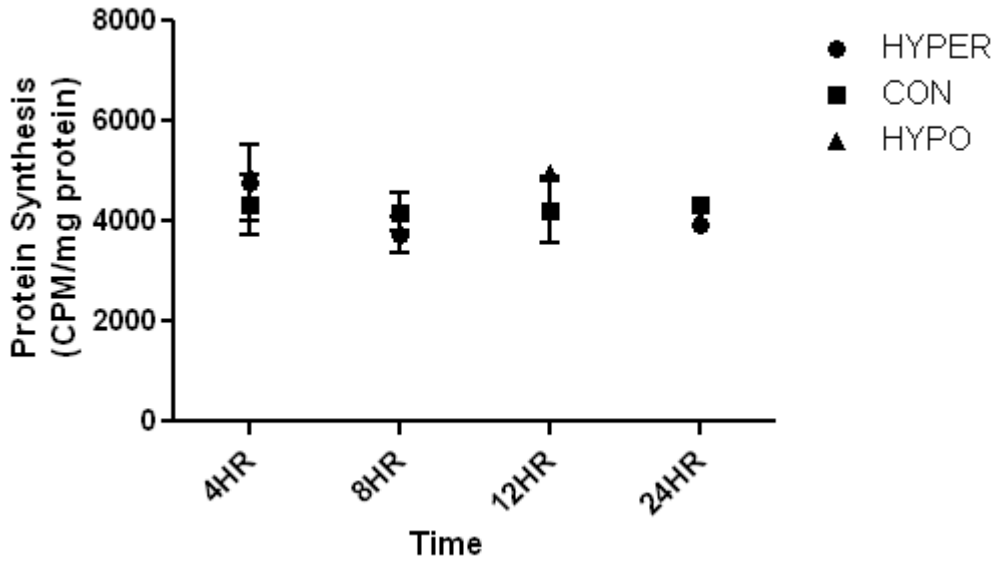


Figure 12. *Protein synthesis measurements after incubation of cells in experimental media for differing amounts of time. Values expressed as mean ± SEM (n=8/group). HYPER=Hyperosmotic stress, CON=Control, HYPO=Hypo-osmotic stress, CPM=Counts per minute.*

III. Western Blotting Analysis

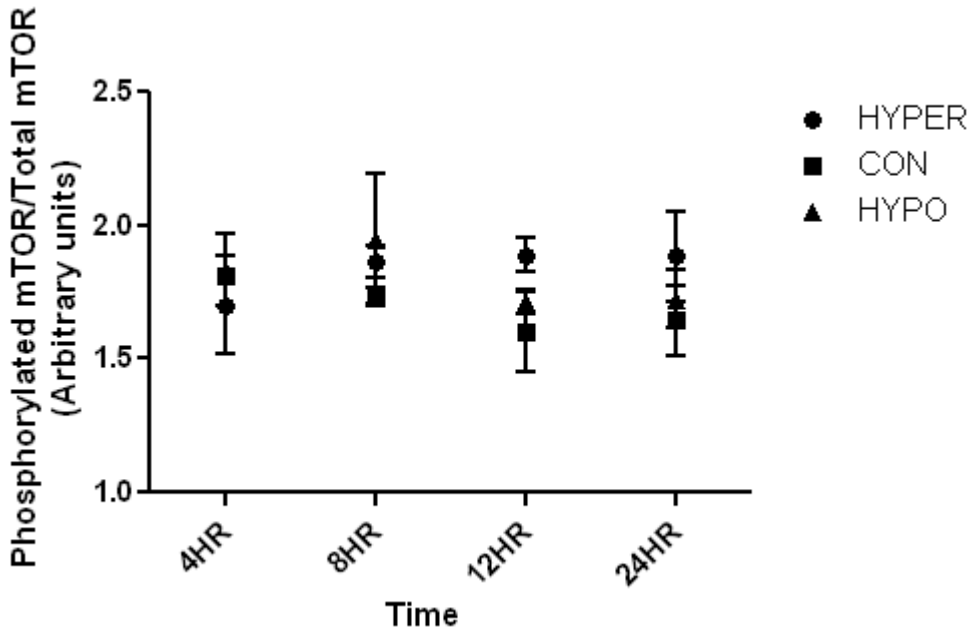
IIIa. Protein Synthesis Markers

There was no significant difference in the ratio of phosphorylated/total mTOR between each condition and thus no significant interaction or main effects for time or osmolality (Figure 13).

In regards to the ratio of phosphorylated p70S6K/total p70S6K, there was

a significant interaction between time and osmolality. There was a significant main effect for time. In general, there was an inverse relationship between time and proportion of phosphorylated p70S6K. Also, there was a significant main effect for osmolality. At each specific time point, HYPO is lower than both HYPER and CON, while HYPER and CON are similar with the exception of the 8hr time point. (Figure 14).

a.



b.

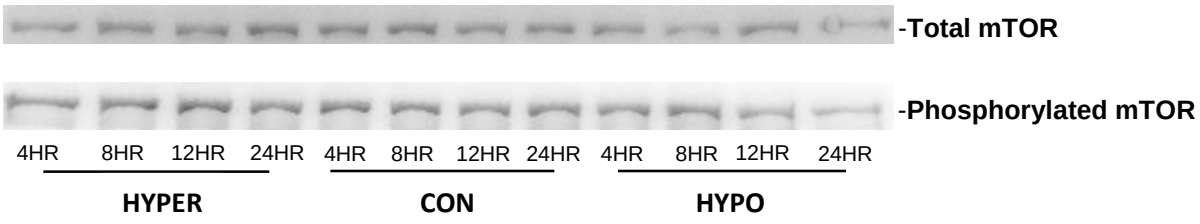
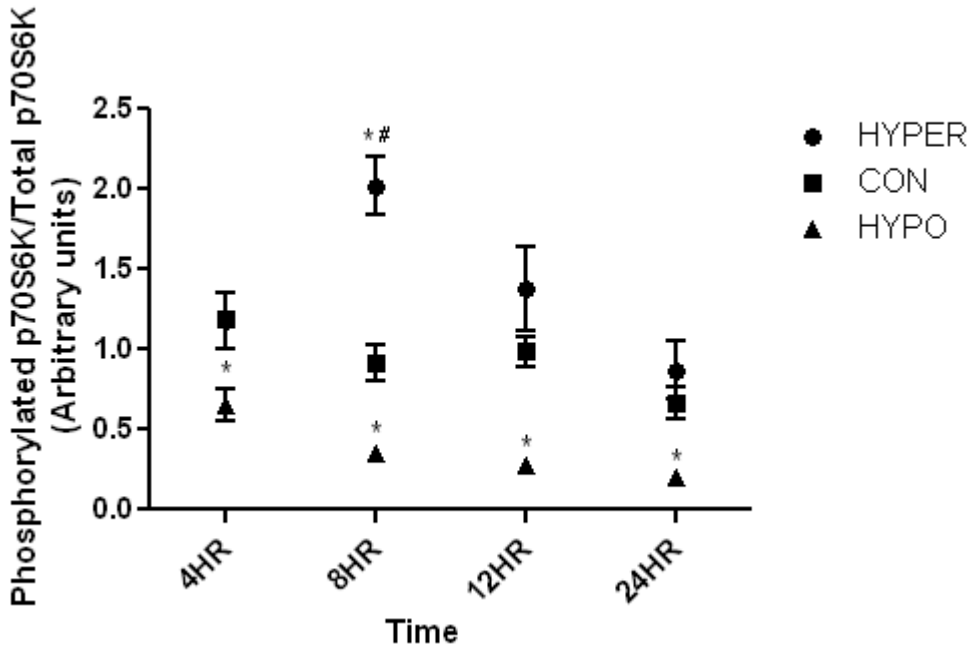


Figure 13. *a) Proportion of phosphorylated mTOR to total mTOR after incubation of cells in experimental media for differing amounts of time, b) Corresponding representative blots. Values expressed as mean ± SEM (n=3/group). HYPER=Hyperosmotic stress, CON=Control, HYPO=Hypo-osmotic stress, mTOR=Mammalian Target of Rapamycin.*

a.



b.

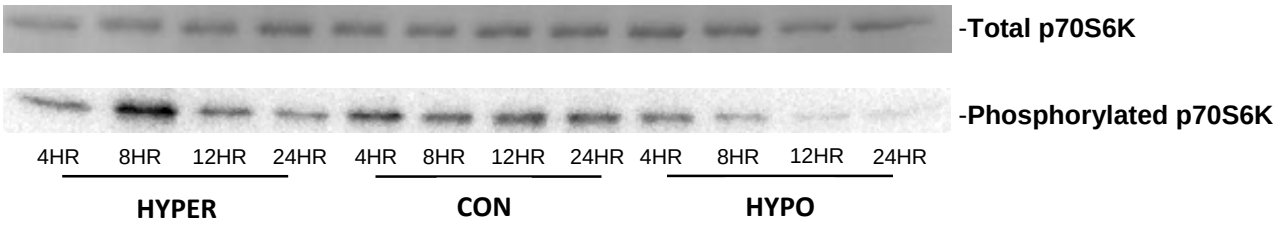


Figure 14. *a) Proportion of phosphorylated p70S6K to total P70S6K after incubation of cells in experimental media for differing amounts of time, b) Corresponding representative blots. Values expressed as mean ± SEM (n=3/group). * denotes a significantly different mean than that of CON at the corresponding time, # denotes a significantly different mean than that of the 4hr time point for the same osmotic condition (p<0.05). HYPER=Hyperosmotic stress, CON=Control, HYPO=Hypo-osmotic stress, p70S6K= p70 Ribosomal S6 Kinase 1*.

IIIb. Protein Degradation Markers

There was no detectable autolysis of μ-calpain for any of the conditions. As such, there was no statistical difference in μ-calpain activation across conditions (Figure 15).

There was also no significant difference in the activation of the proteasomal system as, measured by polyubiquitinated proteins, between any of the groups (Figure 16).

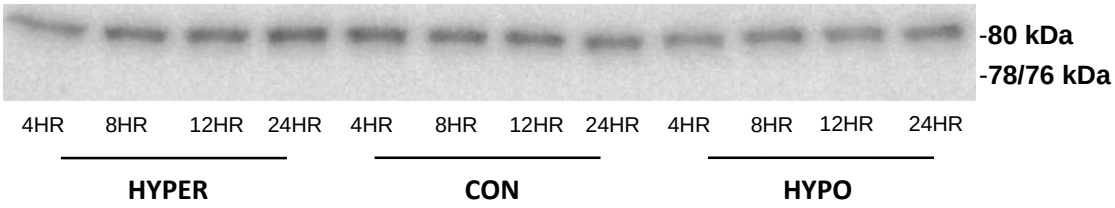
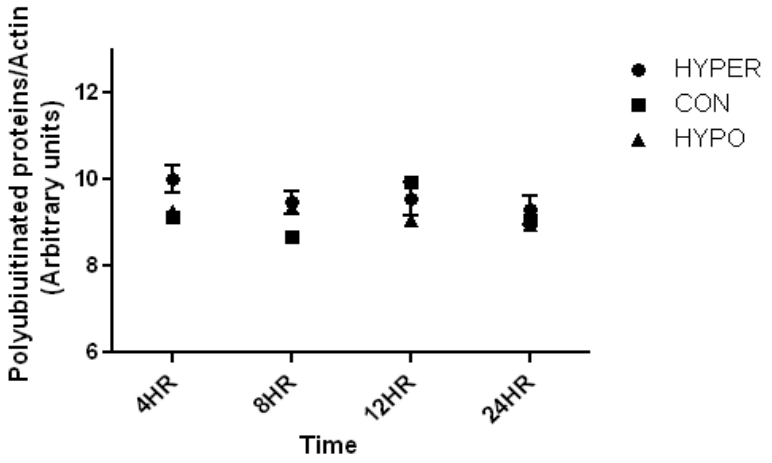


Figure 15. *Representative blot of unautolyzed* μ-calpain (80kDa) and autolyzed μ-calpain (78/76kDa) *(n=2/group). HYPER=Hyperosmotic stress, CON=Control, HYPO=Hypo-osmotic stress.*

a.



b.

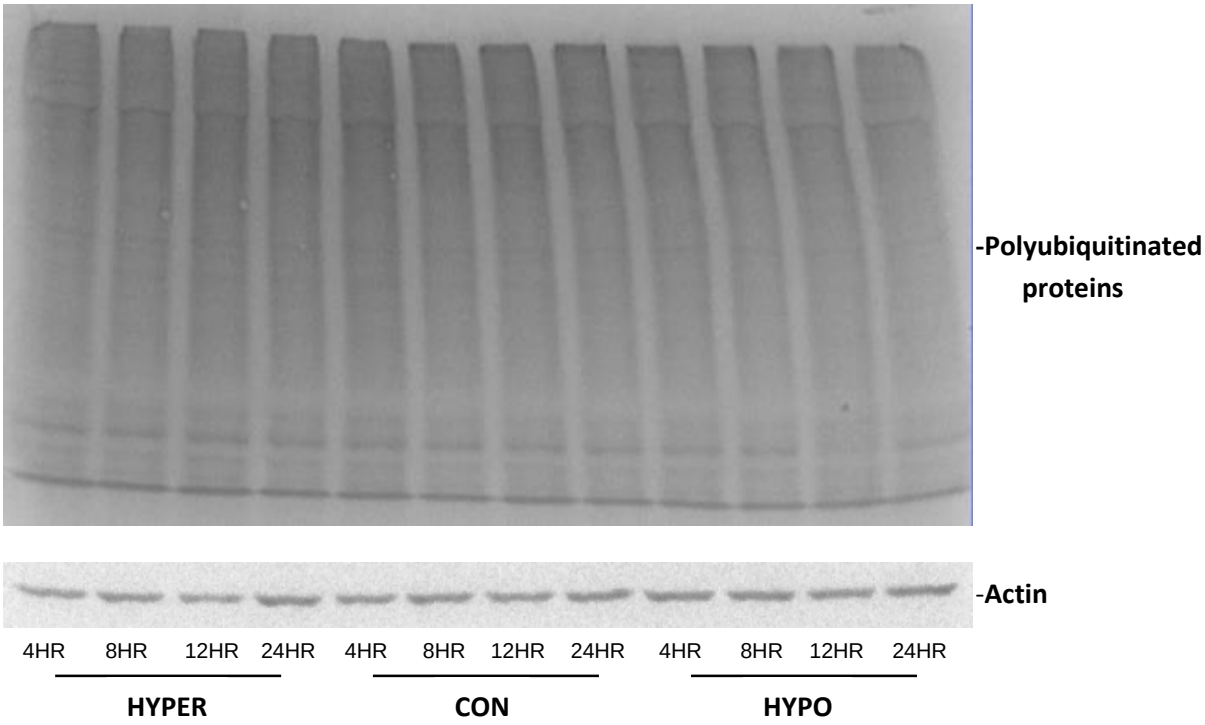


Figure 16. *a) Polyubiquitinated proteins normalized to actin after incubation of cells in experimental media for differing amounts of time, b) Corresponding representative blots. HYPER=Hyperosmotic stress, CON=Control, HYPO=Hypo-osmotic stress.*

Chapter 5: Discussion

Contrary to the initial hypothesis, this study provided evidence that extracellular osmotic stress has minimal impact on protein turnover in L6 skeletal muscle cells independent of the duration or type of osmotic stress. The key finding of this current study was that after exposure to experimental media (HYPER, CON, or HYPO) for varying amounts of time, there were no observed differences in protein synthesis (as estimated by the amount of incorporated [^{3}H]-tyrosine) between the different groups and time points. Supporting this finding was the observation that there were no significant differences between any of the groups in the proportion of phosphorylated mTOR/total mTOR. However, we observed that osmolality and time had a significant interaction on the proportion of phosphorylated p70S6K/total p70S6K. HYPO demonstrated a reduction in p70S6Ks phosphorylation at all time points, while HYPER and CON remained similar except for a large increase observed at 8hrs for HYPER.

Other findings of this study suggest that protein degradation in the current model is unaffected by extracellular osmotic stress, regardless of the duration. This was supported by observations that extracellular osmotic stress did not appear to affect the amount of μ-calpain autolysis or polyubiquitinated proteins.

I. Protein Synthesis

No differences were seen in protein synthesis, as estimated by [^{3}H]-tyrosine incorporation, in response to varying durations (4, 8, 12, or 24 hours) of extracellular osmotic stress. Currently, literature regarding this particular topic is

sparse. However, from the limited studies that have published, it appears that this finding was not in agreement with what others have observed. In general, past studies in skeletal muscle have observed the following to be true; hyperosmotic stress depressed protein synthesis, and hypo-osmotic stress elicited increased levels of protein synthesis (Roy, Russell et al. 2009, Darling, LeBlanc et al. 2011). These past studies are further supported by the long standing theory of cell swelling, which essentially states that the swelling of a cell creates an anabolic environment whereas cell shrinkage produces a catabolic environment (Haussinger 1996). In the case of extracellular osmotic stress, this is thought to occur as a method of attenuating the difference in osmolality across the membrane. More specifically, when cell swelling occurs (due to extracellular hypo-osmotic stress) small molecules such as amino acids are combined to form larger ones, in this case proteins, as a means to decrease the intracellular osmolality and as a result minimize the difference in osmolality across the membrane. The opposite is seen in cases where there is a hyperosmotic extracellular stress; larger molecules will be broken down into their smaller subunits to increase intracellular osmolality. Following this, RVI (HYPER) and RVD (HYPO) processes will occur, triggering an influx and efflux of osmolytes, respectively. Water molecules will subsequently follow the movement of osmolytes, effectively bringing cell volume back towards resting values. This theory has been well documented and has been supported in several models with the majority of evidence being observed in hepatocytes (Haussinger 1993). With this in mind, the observed findings in the current study relating to protein

synthesis appear to be somewhat of an anomaly. There are a few possible explanations for the discrepancy between the current study and literature. One possibility is that the combination of the particular isotope ([^{3}H]) and scintillation counter was not sensitive enough to detect differences. Past studies involving the measurement of the protein synthetic response have used different radioisotopes, which are more accurately and efficiently counted, so it is possible that the methods available limited the sensitivity and thus no alterations in protein synthesis were observed. More specifically, it is common for ^{14}C to be selected for measurements of protein synthesis, as it is more accurately counted (Pace and Manahan 2007, Darling, LeBlanc et al. 2011). Another possible explanation is that proteins which were incorporating the radiolabeled tyrosine, were simultaneously being broken down due to elevated activity levels by one or more of the protein degradation systems. This could have resulted in no significant alterations in protein synthesis being detected.

The Akt/mTOR/p70S6K signalling pathway is thought to play a key role in the regulation of protein synthesis (Miyazaki and Esser 2009). Two of the main proteins that are found in this pathway are mTOR and p70S6K. After mTOR is phosphorylated and effectively activated it will then lead to the subsequent phosphorylation and activation of p70S6K. It is well documented in the literature that upon phosphorylation of these proteins, protein synthesis is upregulated shortly thereafter (Laplante and Sabatini 2009). As there was no significant difference in protein synthesis between any experimental conditions, it was expected that the phosphorylation state of these two proteins would remain

unchanged. As expected, there was no change in the proportion of phosphorylated mTOR. There was, however, unanticipated alterations in the proportion of phosphorylated p70S6K between the different osmotic conditions. More specifically, it was observed that HYPO lead to decreased levels of p70S6K phosphorylation when compared to both CON and HYPER at all observed time points (4, 8, 12, and 24hrs). Whereas CON and HYPER remained relatively similar with the exception of a significant increase in phosphorylation seen for HYPER at 8hrs. Currently, there is only one other known study which has looked at the effects of extracellular osmotic stress on the phosphorylation of p70S6K in skeletal muscle (Darling, LeBlanc et al. 2011). This study was conducted in rat muscle tissue, and it was found that HYPO induced increased levels of phosphorylation while HYPER resulted in depressed levels. Similar studies have been conducted in other models, with the findings generally lending support to the past study done in skeletal muscle, while contradicting the results from the current study. These models have included kidney cells (HEK293), CHO cells, myeloma cells (MPC 11), and hepatocytes (Kruppa and Clemens 1984, Parrott and Templeton 1999, Webster, Blanch et al. 2000, Morley and Naegele 2002, Patel, McLeod et al. 2002). Interestingly, one study has shown increased levels of p70S6K phosphorylation in kidney cells (MDCK) as a result of hyperosmotic stress (Terada, Tomita et al. 1994). This is thought to occur through the MAPK signalling pathway which functions independently from Akt but not mTOR.

With this in mind, it seems quite possible that in the current study p70S6K phosphorylation was elevated in response to hyperosmotic stress through an

upregulation of the MAPK signalling pathway. However, it does not explain why a concurrent increase in mTOR phosphorylation was not seen, despite being a requirement for the phosphorylation and subsequent activation of p70S6K. According to the literature, there are presently no identified mechanisms of p70S6K phosphorylation which occur independently from mTOR phosphorylation. Possible explanations for the dissociation between these two proteins seen in the current study include mTOR phosphorylation occurring and subsiding before the earliest time point, and phosphorylation occurring at a different residue on mTOR than the one investigated (Ser2448). Of particular interest is the postulation that mTOR may have been phosphorylated at a different residue than the one of interest for the current study. To date, four separate phosphorylation sites have been identified on mTOR (Ser2481, Ser2448, Ser1261, and Thr2446) (Acosta-Jaquez, Keller et al. 2009). Ser2448 was selected as the phosphorylation site of interest for the present study as it has been identified as a target site for Akt to exert its downstream effects on mTOR (Chiang and Abraham 2005). However, recently Ser1261 has been shown to have a key role in regulating the activation of mTOR and also appears necessary for downstream activation of p70S6K to occur (Acosta-Jaquez, Keller et al. 2009). With that in mind, mTOR may have been phosphorylated at a different site, possibly Ser1261, and thus activated without detection in the current study. Antibodies for mTOR phosphorylation at Ser1261 are not commercially available, so it is currently not possible to detect phosphorylation at this residue without making your own antibody.

II. Protein Degradation

In contrast to protein synthesis, no direct measurements were conducted to estimate overall protein degradation within the cells. However, markers for two of the common protein degradation systems in skeletal muscle were monitored in response to each of the experimental conditions. These markers included overall autolysis of μ-calpain (to estimate the activity of the μ-calpain system), and the quantity of polyubiquitinated proteins (to estimate the activity of the ubiquitin proteasome system). In the current study, autolysis was not detected in any of the groups and as such no differences were seen in the autolysis of μ-calpain between experimental conditions. This is in stark contrast with the only known study to previously monitor μ-calpain autolysis in response to osmotic stress in skeletal muscle. The previous study, which was in whole muscle, observed increased levels of μ-calpain autolysis in response to both hyperosmotic stress and hypo-osmotic stress (135 minute exposure) (Darling, LeBlanc et al. 2011). In terms of monitoring the activity of μ-calpain in response to osmotic stress, there is currently limited literature regardless of model used. However, there have been several studies which have seen increased calcium transients in response to both HYPO and HYPER osmotic stress. These studies have utilized a multitude of different models including; skeletal muscle, cardiac myocytes, and astrocytes (Schliess, Sinning et al. 1996, Bewick, Fernandes et al. 1999, Apostol, Ursu et al. 2009). As previously mentioned, activation of calpain requires increased levels of intracellular Ca^{2+}, and it seems possible that these increases could lend support to the previous findings in whole muscle.

As for why no alterations in μ-calpain activation were seen in the current study, it could be related to the model that was used. Cell culture models can have decreased amounts of intact microtubules and these may be crucial for causing the dramatic alterations in cell volume and shape seen in whole muscle (Haussinger, Stoll et al. 1994, vom Dahl, Stoll et al. 1995). The working hypothesis is that alterations in cell shape result in changes to the conformation of DHPR and RR in the transverse tubules thus causing an influx of calcium and an activation of calpain (Pickering, White et al. 2009). If it were the case that our current model had a decreased amount of intact microtubules and as a result alterations in cell shape did not occur to a great enough extent, this could have possibly lead to diminished levels of intracellular Ca^{2+}. Reductions in intracellular Ca^{2+} present a possible explanation as to why μ-calpain remained in its inactive state regardless of extracellular osmotic stress.

Similarly to μ-calpain, it was observed that there was no difference in the polyubiquitination of proteins between any of the experimental groups. This is in agreement with the only known study to have used this marker in skeletal muscle as a result of exposure to extracellular osmotic stress (Darling, LeBlanc et al. 2011). Polyubiquitination of proteins has also been used as a marker in HeLa cells in response to hyperosmotic stress (Lee, Park et al. 2010). In this study it was observed that increasing the osmolality of the medium and inducing hyperosmotic stress resulted in a greater amount of polyubiquitinated proteins after 1hr of stress. In comparison, the current study investigated the amount of polyubiquitinated proteins at 4, 8, 12, and 24 hours of osmotic stress. One

possible explanation for the observed disparity could be that the ubiquitination of proteins occurred earlier or later than any of the monitored time points. Polyubiquitination of proteins has been shown to be dependent on both the stress and model used and the time in which it occurs can be variable (Seiffert, Gosenca et al. 2007, Majetschak, Patel et al. 2008). Another potential explanation is that the polyubiquitination of proteins was not reflective of the overall activity of the system in this particular model. This is due to the fact that immunoblotting for ubiquitin conjugates does not take into account any external factors that could alter the overall quantity. Some factors affecting the amount of polyubiquitinated proteins include the activity of E3 ligases, the ability to transport ubiquitinated proteins to the proteasome, and also in the activity of the proteasome itself (Lee, Park et al. 2010). Due to this, it is difficult to compare levels of polyubiquitination across varying models and stresses in which there may be dysregulation in one or more of these factors.

Chapter 6: Conclusion

Exposure of L6 skeletal muscle cells to extracellular hyperosmotic (425.3 ± 1.8mmol/kg) and hypo-osmotic (235.4 ± 1.0mmol/kg) stress had minimal effects on protein synthesis and no observable effects on protein degradation regardless of duration. The findings from this study suggest that these particular levels of extracellular osmotic stress do not significantly affect protein turnover in L6 skeletal muscle cells. In specific reference to the purpose and the initial hypotheses, the following was observed:

1) Neither hyperosmotic nor hypo-osmotic stress induced any alterations in protein synthesis as measured by [^{3}H] labeled tyrosine incorporation.

2) Neither hyperosmotic nor hypo-osmotic stress induced any alterations in the ubiquitin proteasome and calpain protein degradation pathways. As measured by the amount of polyubiquitinated proteins and autolysis of μ-calpain, respectively.

3) Hyperosmotic and hypo-osmotic stress had slight influences on the Akt/mTOR/p70S6K protein synthesis signalling pathway. The pathway remained unaltered in response to hyperosmotic stress with the exclusion of a large increase in p70S6K phosphorylation seen at 8hours. In contrast, hypo-osmotic stress lead to decreased levels of p70s6k phosphorylation at all time points. No alterations were observed for with stress in terms of mTOR phosphorylation.

Figure 17 provides a summary of the conclusions.

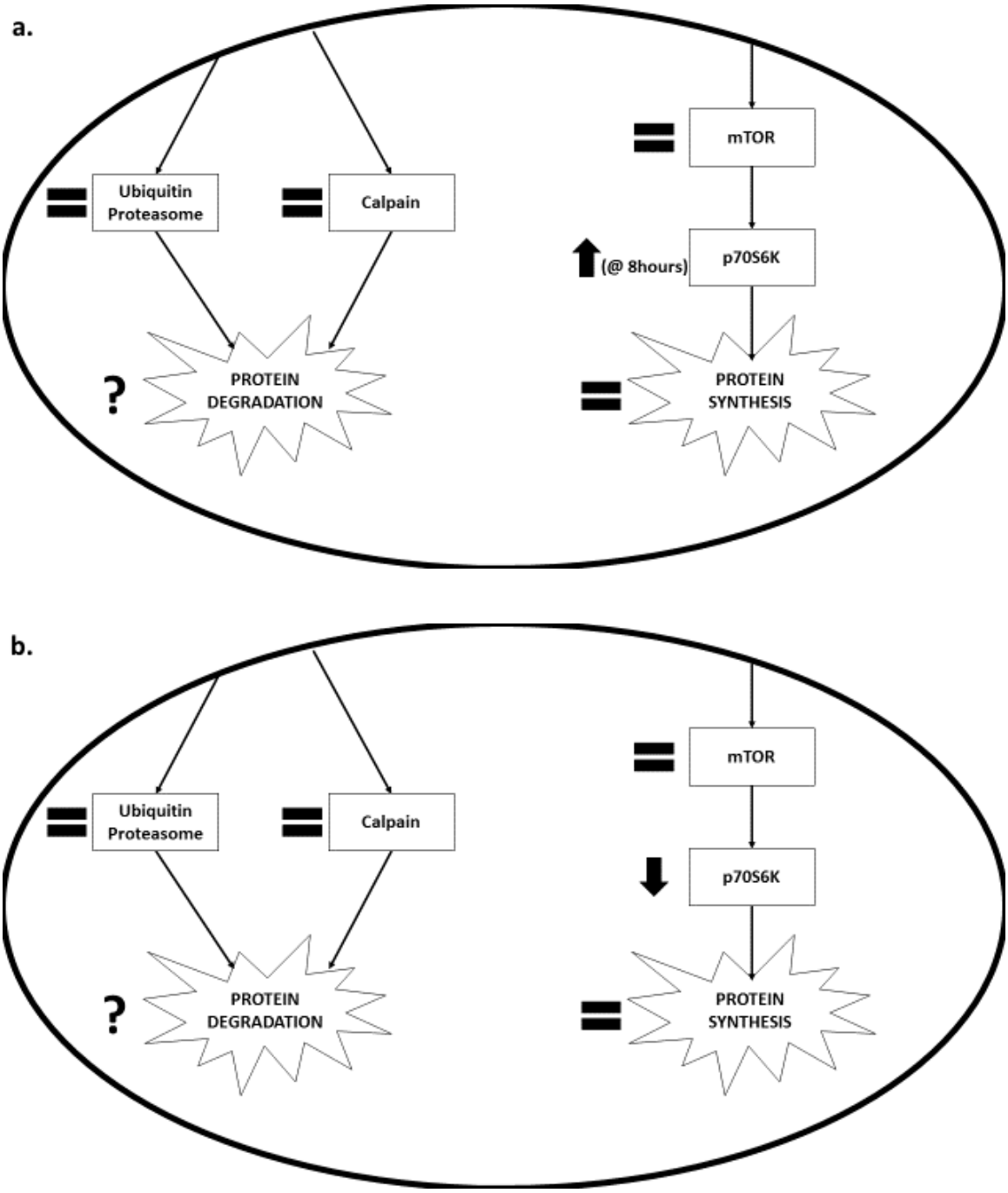


Figure 17. *Visual representation of observed outcomes in response to; a) hyperosmotic stress, b) hypo-osmotic stress. mTOR = mammalian target of rapamycin, p70S6K = p70 Ribosomal S6 Kinase,* 〓 *= no change.*

Chapter 7: Strengths and Limitations

I. Strengths

The current study was one of only a handful of studies that have investigated the effects of extracellular osmotic stress on protein metabolism in skeletal muscle, and was the first to examine the influence of time of stress exposure. A strength of this study was the utilization of an L6 skeletal muscle cell culture model. In comparison to whole muscle, cell culture offered a model which was easily modified and controlled, with a greater amount of accessible surface area. This allowed for the rapid uptake of nutrients and substrates, allowing for the measurement of metabolic processes while maintaining cell survivability for extended periods of time. It also allowed for the measurement of a large number of samples while maintaining reproducibility due to similarities in the genotype of cells across all specimens.

This leads to the next major strength of this research. Experiments were all conducted on specimens at similar stages of life, while passaging and feeding of cells were also maintained on similar schedules across all samples. More specifically the following was done to ensure reproducibility and minimize error: 1) cells were passaged three times after thawing to ensure viability; 2) cells were plated at a density of 100000 cells/well and allowed to proliferate for 24hrs; and 3) all experiments were conducted after 7 days of differentiation at passage 7. This ensured that all samples were treated the exact same way and that any effects which were observed were due to the experimental protocol and not fundamental differences in the samples themselves.

II. Limitations

Limitations of this study are mainly related to the equipment and infrastructure available at the time of experimentation. As previously mentioned there are strengths to the use of cell culture as a model. But there are also limitations. Cell culture has been noted to not be representative of what would truly happen in the human body, and caution must be taken when applying these findings to humans or other mammals. This is due to a lack of integration with other systems in the body which would normally act to regulate the variables being monitored.

The use of the radioisotope tritium [^{3}H] was another possible limitation of the study as it may not be the most sensitive radioisotope for the measurements performed in the current study based on the scintillation counter used. The scintillation counter used for this study had a counting efficiency for tritium of ~65%, meaning that 35% of the released photons from the tritiated proteins were not being counted. Tritium is found on the side chain of an amino acid, and it has been shown that this label can be lost due to intracellular reactions with water (Williams, Sugden et al. 1981). This may have resulted in significantly decreased CPM readings. Conversely, another radioisotope commonly used for estimating protein synthesis, radiocarbon [^{14}C], has a quoted efficiency of ~98%. The increased efficiency means that the measurements from the scintillation counter would be more representative of what was actually occurring. However, radioisotopes with higher counting efficiencies are often associated with greater costs and/or higher safety risks due to higher levels of energy released upon

decay.

Lastly, the absence of a true measurement or estimation of protein degradation was another limitation. Several assay kits are available to measure the activity level of the different degradation systems commonly found in muscle, however; for this particular study they were not compatible due to the experimental timeline and cell harvesting procedures utilized in this study. Instead, the autolysis of μ-calpain and polyubiquitination of proteins were used as markers for the activity of the calpain and ubiquitin proteasome degradation systems, respectively. However; as previously mentioned the polyubiquitination of proteins is not necessarily an accurate measurement of the activity of the degradation system due to several other external influences which can alter the rate of appearance of polyubiquitinated proteins. Autolysis of μ-calpain also may not have been a reliable marker of protein degradation as the model selected may not reach the intracellular Ca^{2+} threshold needed to activate this degradation system.

Chapter 8. Future Directions

Future research should be conducted to confirm the findings of the current study, as there was a significant disparity between current and past studies. The following should be considered when designing future studies to confirm these findings:

i. The use of alternative radioisotopes to measure protein synthesis

As mentioned, tritium only had a counting efficiency of ~65%. To increase the sensitivity when measuring protein synthesis other viable options should be considered. These options include; ^{14}C (98% counting efficiency), and $^{32}P/^{33}P/^{35}S/^{125}I$ (100% counting efficiency). Taking into consideration the relative safety and cost of using each alternative radioisotope, the optimal choice is ^{14}C. This radioisotope will also minimize the loss of label that may be seen with tritium, because ^{14}C is incorporated into the protein as part of the backbone and not a sidechain.

ii. Conducting a relative measurement of protein degradation or activity of specific degradation systems.

Future studies could estimate protein degradation by measuring the appearance of tyrosine after the addition of cycloheximide as previously described (Dardevet, Sornet et al. 1994). The relative activity of different protein degradation systems could be measured utilizing fluorometry assay kits. These kits use different fluorophores which will emit a certain spectrum of light in response to cleavage by certain systems. More specifically, the AFC (7-amino-4-trifluoromethylcoumarin) and AMC (7-amino-4-methylcoumarin) fluorophores,

which have been shown to measure the activity of calpain and overall proteolysis, respectively (Pickering and Davies 2012, Cui, Ma et al. 2013). These measurements would help to validate and clarify the results found from analysis of the markers of protein degradation.

iii. Investigation of upstream/downstream proteins, and alternative signalling pathways

Observing the state and activation of several downstream and upstream proteins along the Akt/mTOR/p70S6K signalling pathway will further elucidate the mechanisms behind alterations in protein synthesis, if any are observed. These proteins could include Akt, eIF4E, and PDCD4. Another intriguing avenue is to monitor some of the proteins in the MAPK pathway. As previously stated, p70S6K has been shown to be upregulated in response to hyperosmotic stress, and this is thought to have occurred through the MAPK pathway (Terada, Tomita et al. 1994). It would then be possible to investigate how these two pathways respond to extracellular osmotic stress and such knowledge would further our understanding on the topic.

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Appendix

Immunoblotting Protocol for Specific Proteins

	Anabolic Markers				Catabolic Markers		Other
Protein	mTOR	p-mTOR	p70S6K	p-p70S6K	Ubiquitin	µ-Calpain	Actin
Sample Load	5 µg	5 µg	10 µg	10 µg	10 µg	10 µg	10 µg
Stacking Gel	4%	4%	4%	4%	4%	4%	4%
Running Gel	7.5%	7.5%	10%	10%	7.5%	7.5%	7.5%
Electrophoresis	150min @ 100V (on ice)	150min @ 100V (on ice)	90min @ 120V	90min @ 120V	90min @ 100V	90min @ 120V	90min @ 100V
Membrane	0.45µm PVDF	0.45µm PVDF	0.2µm PVDF	0.45µm PVDF	0.45µm PVDF	0.45µm PVDF	0.45µm PVDF
Transfer	120min @ 100V	120min @ 100V	90min @ 100V	90min @ 100V	75min @ 100V	90min @ 100V	75min @ 100V
Blocking	5% milk/TBST for 1hr	5% milk/TBST for 1hr	2.5% milk/TBST for 1hr	5% milk/TBST for 1hr	5% milk/TBST for 1hr	5% milk/TBST for 1hr	5% milk/TBST for 1hr
1° Antibody	1:1000 rabbit polyclonal in 5%BSA/TBST overnight	1:1000 rabbit polyclonal in 5%BSA/TBST overnight	1:1000 rabbit polyclonal in 5%milk/TBST overnight	1:1000 rabbit polyclonal in 5%BSA/TBST overnight	1:1000 mouse monoclonal in 5%milk/TBST overnight	1:1000 rabbit polyclonal in 5%BSA/TBST overnight	1:1000 goat polyclonal in 5%milk/TBST overnight
2° Antibody	1:5000 anti-rabbit in 5%milk/TBST for 1 hr	1:5000 anti-rabbit in 5%milk/TBST for 1 hr	1:5000 anti-rabbit in 5%BSA/TBST for 1 hr	1:5000 anti-rabbit in 5%milk/TBST for 1 hr	1:5000 anti-mouse in 5%milk/TBST for 1 hr	1:5000 anti-rabbit in 5%milk/TBST for 1 hr	1:5000 anti-goat in 5%milk/TBST for 1 hr
Exposure	2 min	1 min	2 min	1 min	1 min	1 min	1 min